A behavioural evaluation of the potential of nNOS inhibitors to control dyskinesia in animal models of Parkinson's disease

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A behavioural evaluation of the potential of nNOS inhibitors to control dyskinesia in animal models of Parkinson's disease

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Certificate

This is to certify that I have carried out the studies embodied in this thesis under the supervision and guidance of Dr Sarah Salvage and Professor Peter Jenner.

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Abstract

Long-term dopaminergic therapy in Parkinson's disease (PD) can lead to motor complications including dyskinesia which can be treated with amantadine, an N-methyl-D-aspartate (NMDA) receptor antagonist. NMDA receptor activation is linked with nitric oxide (NO) production and changes in synaptic plasticity, suggesting a role in dyskinesia. This led to the hypothesis that NO, produced by neuronal nitric oxide synthase (nNOS), contributes to the occurrence and evolution of dyskinesia in PD. Therefore, these studies investigated the effects of nNOS inhibition, using the nNOS inhibitors ARR17477 and 7-nitroindazole (7-NI), on the dyskinesia expression and priming processes in rodent and primate models of PD following L-dopa and dopamine agonist treatment.

To explore the role of nNOS inhibition on established dyskinesia, 6-OHDA-lesioned rats, primed to exhibit stable abnormal involuntary movements (AIMs), the rodent analogue of dyskinesia, were acutely challenged with nNOS inhibitors plus L-dopa or ropinirole. No reduction in AIMs was observed following nNOS inhibition.

In order to investigate the potential for nNOS inhibitors to reduce the priming for AIMs, naïve 6-OHDA-lesioned rats were treated chronically with ARR17477 or 7-NI plus either L-dopa or ropinirole. Again, there was no beneficial effect of nNOS inhibition on the emergence of L-dopa- or ropinirole-induced AIMs.

nNOS inhibition was also investigated in MPTP-treated primates, the gold standard behavioural model of PD. ARR17477 did not reduce the expression of established dyskinesia following L-dopa or ropinirole treatment. Similarly nNOS inhibition did not attenuate L-dopa-induced priming for dyskinesia in this model.

In conclusion, inhibition of nNOS in 6-OHDA-lesioned rats and MPTP-treated primates did not reduce the expression or priming of L-dopa- or ropinirole-induced dyskinesia. These findings do not support a role for nitric oxide in processes underlying dyskinesia and suggest that nNOS inhibitors would not be beneficial in either preventing or attenuating motor complications in PD patients.

Table of contents

Certificate **2**

Abstract **3**

Table of contents **4**

Table of figures **9**

Table of tables **12**

List of abbreviations **13**

Publications **15**

Acknowledgements **16**

Chapter 1 : General introduction **17**

1.1 Parkinson's disease **18**

1.1.1 Aetiology of PD 18

1.1.2 Pathology of PD 19

1.1.3 Clinical features of PD 20

1.2 PD therapy **21**

1.2.1 L-dopa 21

1.2.2 Dopamine agonists 22

1.2.3 L-dopa, dopamine agonists and motor complications 23

1.3 Dyskinesia **24**

1.3.1 Risk factors 25

1.3.2 Pathophysiology of dyskinesia 25

1.3.3 Experimental animal models of PD and dyskinesia 29

1.3.4 Therapeutic options addressing dyskinesia 31

1.4 NO, nNOS and nNOS inhibitors **32**

1.4.1 Synthesis of NO 32

1.4.2 NO, the basal ganglia and synaptic plasticity 35

1.4.3 NOS Inhibitors 37

1.4.4 NOS in PD and dyskinesia 39

1.5 Thesis hypothesis **41**

1.6 Thesis aims **41**

Chapter 2 : Materials and methods **42**

2.1 Introduction **43**

2.2 The 6-OHDA rat model: **43**

2.2.1 Introduction 43

2.2.2 Animal husbandary 43

2.2.3 6-OHDA lesion induction 44

2.2.4 Post-operative care 45

2.3 The 6-OHDA rat model: Behavioural assessment 45
2.3.1 Automated measurement of rotational activity 45
2.3.2 Dyskinesia assessment 46
2.4 The MPTP-treated primate model: 59
2.4.1 Introduction 59
2.4.2 Animal husbandary 59
2.4.3 MPTP-lesion induction 59
2.4.4 Post-MPTP care 59
2.4.5 Behavioural effects of MPTP administration 60
2.5 The MPTP-treated primate model: Behavioural assessment 60
2.5.1 Priming for dyskinesia 60
2.5.2 Locomotor activity measurement 61
2.5.3 Motor disability assessment 61
2.5.4 Dyskinesia assessment 61
2.5.5 Establishing doses for L-Dopa and ropinirole 62
2.6 Biochemical Techniques 66
2.6.1 Radioenzymatic measurement of NOS activity in brain tissue 66
2.6.2 Determination of tyrosine hydroxylase 70
2.7 General materials used 72

Chapter 3 : The effects of nNOS inhibitor treatment on expression of AIMs in 6-OHDA-lesioned rats 74

3.1 Introduction 75
3.1.1 Hypothesis 76
3.1.2 Aims 76
3.2 Materials and methods 77
3.2.1 Introduction 77
3.2.2 Animals 77
3.2.3 Ex vivo NOS assay 77
3.2.4 Assessment of the effect of nNOS inhibition on AIMs in L-dopa primed 6-OHDA-lesioned rats 81
3.2.5 Data and statistical analysis 83
3.3 Results 84
3.3.1 Locomotive AIMs in L-dopa-primed 6-OHDA-lesioned rats treated acutely with L-dopa plus nNOS inhibitor 84
3.3.2 Locomotive AIMs in L-dopa-primed 6-OHDA-lesioned rats treated acutely with ropinirole plus nNOS inhibitor 84
3.3.3 Axial, limb, orolingual and ALO AIMs in L-dopa-primed 6-OHDA-lesioned rats treated acutely with L-dopa plus nNOS inhibitor 90

3.3.4 Axial, limb, orolingual and ALO AIMs in L-dopa-primed 6-OHDA-lesioned rats treated acutely with ropinirole plus nNOS inhibitor 96
3.4 Discussion 102
3.4.1 Inhibition of nNOS by ARR17477 and 7-NI 102
3.4.2 The effect of nNOS inhibition on L-dopa-induced AIMS expression 103
3.4.3 The effect of nNOS inhibition on ropinirole-induced AIMS expression 105
3.4.4 Conclusion 106

Chapter 4 : The effects of nNOS inhibitor treatment on priming for AIMs in 6-OHDA-lesioned rats 107
4.1 Introduction 108
4.1.1 Hypothesis 109
4.1.2 Aims 109
4.2 Materials and methods 110
4.2.1 Introduction 110
4.2.2 Animals 110
4.2.3 Determination of the dose of nNOS inhibitor 110
4.2.4 Behavioural Studies 113
4.2.5 Data and statistical analysis 114
4.3 Results 117
4.3.1 AIMs in 6-OHDA-lesioned rats primed with nNOS inhibitor alone 117
4.3.2 Locomotive AIMs in 6-OHDA-lesioned rats chronically treated with L-dopa plus nNOS inhibitor 118
4.3.3 Locomotive AIMs in 6-OHDA-lesioned rats with ropinirole plus nNOS inhibitor 118
4.3.4 Axial, limb, orolingual and ALO AIMs in 6-OHDA-lesioned rats chronically treated with L-dopa plus nNOS inhibitor 124
4.3.5 Axial, limb, orolingual and ALO AIMs in 6-OHDA-lesioned rats chronically treated with ropinirole plus nNOS inhibitor 130
4.4 Discussion 136
4.4.1 Inhibition of nNOS by chronic treatment with 7-NI 136
4.4.2 Inhibition of nNOS by chronic treatment with ARR17477 136
4.4.3 The effect of chronic nNOS inhibition on L-dopa-induced AIMS 137
4.4.4 The effect of chronic nNOS inhibition on ropinirole-induced AIMS 139
4.4.5 Conclusion 141

Chapter 5 : The effects of nNOS inhibitor treatment on expression and priming for dyskinesia in MPTP-treated primates 143
5.1 Introduction 144
5.1.1 Hypothesis 145

5.1.2 Aims 145
5.2 Materials and methods 146
5.2.1 Introduction 146
5.2.2 Determination of the dose of nNOS inhibitor 146
5.2.3 Animals 147
5.2.4 MPTP-treatment 147
5.2.5 Expression studies 149
5.2.6 Priming study 149
5.2.7 Behavioural assessment 150
5.2.8 Data and statistical analysis 150
5.3 Results 153
5.3.1 The effect of ARR17477 on L-dopa-induced locomotor activity, motor disability and dyskinesia expression in L-dopa-primed MPTP-treated marmosets 153
5.3.2 The effect of ARR17477 on ropinirole-induced locomotor activity, motor disability and dyskinesia expression in L-dopa-primed MPTP-treated marmosets 158
5.3.3 The effect of nNOS inhibition on locomotor activity, motor disability and dyskinesia induction following *de novo* chronic treatment with L-dopa plus nNOS inhibitor in MPTP-treated marmosets 163
5.4 Discussion 169
5.4.1 Inhibition of nNOS by ARR17477 169
5.4.2 Inhibition of nNOS by 7-NI 169
5.4.3 The effect of acute nNOS inhibition on L-dopa-expressed behaviours in MPTP-treated marmosets 170
5.4.4 The effect of acute nNOS inhibition on ropinirole-induced behaviour in MPTP-treated marmosets 172
5.4.5 The effect of chronic nNOS inhibition on L-dopa-induced behaviour in MPTP-treated marmosets 173
5.4.6 Conclusion 175

Chapter 6 : General discussion 177
6.1 Summary and discussion of findings 178
6.1.1 Dyskinesia expression and NO 178
6.1.2 Dyskinesia priming and NO 178
6.1.3 Specificity of NOS inhibitors 180
6.1.4 The animal models 181
6.2 Is there a role for glutamate derived NO in dyskinesia? 184
6.3 What does cause the priming and ongoing expression of dyskinesia? 186
6.4 Future outlook for treatment and prevention of dyskinesia 187
6.5 Final conclusion 188

Appendix.....**189**

References.....**197**

Table of figures

Figure 1-1 Dopamine pharmacology and management of PD 22
Figure 1-2 The basal-ganglia–thalamocortical circuits 27
Figure 1-3 The NOS pathway. ..... **Error! Bookmark not defined.**
Figure 1-4 Domain arrangement in rat nNOS 35
Figure 1-5 Schematic diagram of convergent action of dopaminergic, glutamatergic and nNOS transmission on medium spiny striatal neurones. 36
Figure 2-1 Co-ordinates used for microinjection of 6-OHDA into the MFB 44
Figure 2-2 Tracksys video monitoring of rotational rat behaviour 45
Figure 2-3 Rotations per minute (RPM) typical data. 46
Figure 2-4 AIMs development during L-dopa priming..... 46
Figure 2-5 Transparent cages set up for measuring AIMs in 6-OHDA lesioned rats..... 47
Figure 2-6 The four subtypes of AIMs 48
Figure 2-7 Amantadine AIMs characterisation axial, limb, orolingual and ALO data 52
Figure 2-8 Amantadine AIMs characterisation locomotive data 53
Figure 2-9 MK-801 and 8-OHDPAT AIMs characterisation axial, limb, orolingual and ALO data. 54
Figure 2-10 MK-801 and 8-OHDPAT AIMs characterisation locomotive data 55
Figure 2-11 L-dopa dose-response ALO and locomotive AIMs data t. 57
Figure 2-12 Ropinirole dose-response ALO and locomotive AIMs data. 58
Figure 2-13 Dyskinesia development during L-dopa priming..... 60
Figure 2-14 Behavioural assessment in MPTP-treated marmosets following L-dopa..... 64
Figure 2-15 Behavioural assessment in MPTP-treated marmosets following ropinirole..... 65
Figure 2-17 Protein content standard curve from Nanodrop. 68
Figure 2-16 Example of protein measurement using the NanoDrop spectrophotometer 68
Figure 2-18 Example of radioenzymatic measurement of nNOS activity in brain homogenates 69
Figure 2-19 TH Immunohistochemistry of 6-OHDA-lesioned rat brain section 71
Figure 3-1 Radioenzymatic measurement of the effect of ARR17477 (3, 6 or 12 mg/kg s.c.) on nNOS activity in cerebellum and striatum..... 79
Figure 3-2 Radioenzymatic measurement of the effect of 7-NI (12.5, 25 or 50 mg/kg i.p.) on nNOS activity in cerebellum and striatum..... 80
Figure 3-3 Summary diagram of animal groups and treatments 82
Figure 3-4 Locomotive AIMs expression following ARR17477 plus L-dopa treatment 86
Figure 3-5 Locomotive AIMs expression following 7-NI plus L-dopa treatment 87
Figure 3-6 Locomotive AIMs expression following ARR17477 plus ropinirole treatment 88
Figure 3-7 Locomotive AIMs expression following 7-NI treatment plus ropinirole 89
Figure 3-8 Time-course and Total score for axial (a & a'), limb (b & b'), orolingual (c & c') and ALO AIMs (d & d') expression following ARR17477 plus L-dopa treatment..... 92

Figure 3-9 Peak scores and Duration of activity for axial (a & a'), limb (b & b'), orolingual (c & c') and ALO AIMs (d & d') expression following ARR17477 plus L-dopa treatment 93
Figure 3-10 Time-course and Total score for axial (a & a'), limb (b & b'), orolingual (c & c') and ALO AIMs (d & d') expression following 7-NI plus L-dopa treatment 94
Figure 3-11 Peak scores and Duration of activity for axial (a & a'), limb (b & b'), orolingual (c & c') and ALO AIMs (d & d') expression following 7-NI plus L-dopa treatment 95
Figure 3-12 Time-course and Total score for axial (a & a'), limb (b & b'), orolingual (c & c') and ALO AIMs (d & d') expression following ARR17477 plus ropinirole treatment 98
Figure 3-13 Peak scores and Duration of activity for axial (a & a'), limb (b & b'), orolingual (c & c') and ALO AIMs (d & d') expression following ARR17477 plus ropinirole treatment 99
Figure 3-14 Time-course and Total score for axial (a & a'), limb (b & b'), orolingual (c & c') and ALO AIMs (d & d') expression following 7-NI plus ropinirole treatment 100
Figure 3-15 Peak scores and Duration of activity for axial (a & a'), limb (b & b'), orolingual (c & c') and ALO AIMs (d & d') expression following 7-NI plus ropinirole treatment 101
Figure 4-1 Radioenzymatic measurement of the effect of repeated ARR17477 (0.5, 1, 3 or 6 mg/kg s.c.) treatment on nNOS activity in cerebellum. 112
Figure 4-2 Radioenzymatic measurement of the effect of repeated ARR17477 (1 mg/kg s.c.) treatment on nNOS activity in cerebellum 112
Figure 4-3 Summary time-line of animal groups and priming treatments for chronic AIMs studies ... 116
Figure 4-4 Locomotive (a) and ALO Total AIMs (b) following chronic treatment with ARR17477 or 7-NI 117
Figure 4-5 Locomotive AIMs following chronic treatment with ARR17477 or 7-NI plus L-dopa. 120
Figure 4-6 Locomotive AIMs following final L-dopa challenges. 121
Figure 4-7 Locomotive AIMs following chronic treatment with ARR17477 or 7-NI plus ropinirole.. ... 122
Figure 4-8 Locomotive AIMs following final ropinirole challenges. 123
Figure 4-9 Total and Summed total scores for axial (a-a'), limb (b-b'), orolingual (c-c') and ALO AIMs (d-d') following chronic treatment with ARR17477 or 7-NI plus L-dopa 126
Figure 4-10 Peak and Summed peak scores for axial (a-a'), limb (b-b'), orolingual (c-c') and ALO AIMs (d-d') following chronic treatment with ARR17477 or 7-NI plus L-dopa 127
Figure 4-11 Duration and Summed duration of activity scores for axial (a-a'), limb (b-b'), orolingual (c-c') and ALO AIMs (d-d') following chronic treatment with ARR17477 or 7-NI plus L-dopa 128
Figure 4-12 Final L-dopa challenge data for axial (a-a''), limb (b-b''), orolingual (c-c'') and ALO AIMs (d-d''). 129
Figure 4-13 Total and Summed total scores for axial (a-a'), limb (b-b'), orolingual (c-c') and ALO AIMs (d-d') following chronic treatment with ARR17477 or 7-NI plus ropinirole. 132
Figure 4-14 Peak and Summed peak scores for axial (a-a'), limb (b-b'), orolingual (c-c') and ALO AIMs (d-d') following chronic treatment with ARR17477 or 7-NI plus ropinirole 133
Figure 4-15 Duration and Summed duration of activity scores for axial (a-a'), limb (b-b'), orolingual (c-c') and ALO AIMs (d-d') following chronic treatment with ARR17477 or 7-NI plus ropinirole .. 134

Figure 4-16 Final ropinirole challenge data for axial (a-a''), limb (b-b''), orolingual (c-c'') and ALO AIMs (d-d'') 135
Figure 5-1 Radioenzymatic measurement of the effect of 7-NI (20 mg/kg s.c.) on nNOS activity in cerebellum and striatum 148
Figure 5-2 Summary time-line of animal groups and treatments for priming study 152
Figure 5-3 Locomotor activity following ARR17477 plus L-dopa treatment 155
Figure 5-4 Motor disability following ARR17477 plus L-dopa treatment 156
Figure 5-5 Dyskinesia expression following ARR17477 plus L-dopa treatment 157
Figure 5-6 Locomotor activity following ARR17477 plus ropinirole treatment 160
Figure 5-7 Motor disability following ARR17477 plus ropinirole treatment 161
Figure 5-8 Dyskinesia expression following ARR17477 plus ropinirole treatment 162
Figure 5-9 Locomotor activity following chronic treatment with nNOS inhibitor plus L-dopa 165
Figure 5-10 Motor Disability following chronic treatment with nNOS inhibitor plus L-dopa 166
Figure 5-11 Dyskinesia following chronic treatment with nNOS inhibitor plus L-dopa 167
Figure 5-12 Final L-dopa only challenge data subsequent to chronic nNOS inhibitor plus L-dopa treatment 168
Figure 6-1 Schematic representation of mGluRs at the synapse 185
Figure 0-1 Modified latin-square design for treatment Chapter 3 191
Figure 0-2 Modified latin-square design for treatment Chapter 5 192

Table of tables

Table 1-1 Receptor affinities of dopamine agonists 23
Table 2-1 AIMs classification 48
Table 2-2 AIMs scoring scale 49
Table 2-3 Motor disability scoring classification 61
Table 2-4 Dyskinesia scoring classification 62
Table 2-5 Reagents for radioenzymatic NOS assay 67
Table 2-6 List of drugs 72
Table 2-7 List of chemicals and reagents 72
Table 2-8 List of equipment and consumables 73
Table 4-1 AIMs assessment treatment groups and drug administration 114
Table 0-1 Results of radioligand binding assays for ARR17477 190
Table 0-2 Tables of statistics 193

List of abbreviations

5-HT	5-Hydroxytryptamine (serotonin)
6-OHDA	6-Hydroxydopamine
7-NI	7-Nitroindazole
8-OHDPAT	(+)-8-Hydroxy-2propylaminotetralin
AADC	Aromatic l-amino acid decarboxylase
ABC	Avidin biotin complex
AIMs	Abnormal involuntary movements
ALO AIMs	Axial, limb and orolingual AIMs
AMPA	Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	Analysis of variance
ARR17477	*N*-[4-(2-[(3-Chlorophenyl)methyl]-aminoethyl)phenyl]-2-thiophenecarboximidamide hydrochloride
AUC	Area under the curve
BBB	Blood-brain barrier
BH_4	Tetrahydrobiopterin
BSA	Bovine serum albumin
CAL	calmodulin
cAMP	Cyclic AMP
CNS	Central nervous system
COMT	Catechol-O-methyl transferase
Cpm	Counts per minute
D1/D2/D3	D1/D2/D3 dopamine receptors
DA	Dopamine
DAB	3,3'-diaminobenzidine
DARPP-32	DA- and cAMP-regulated phosphoprotein of 32 KDa
DAT	Dopamine active transporter
DDC	Dopadecarboxylase
DMSO	Dimethyl sulphoxide
DOPAC	3,4 dihydrohylphenylacetic acid
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
ERK1/2	Extracellular signal-regulated kinases 1 and 2
FAD	Flavin adenine dinucleotide
FMN	Flavin adenine mononucleotide
GABA	G-amino‑butyric acid
GDNF	Glial-derived neurotrophic factor
GPe	Globus pallidus pars externa

GPi	Globus pallidus pars interna
HVA	Homovanillic acid
H_2O_2	Hydrogen peroxide
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
L-Dopa	L-3,4-dihydroxyphenylalanine-methyl-ester
LRRK2	Leucine-rich repeat kinase 2
LTD/P	Long-term depression/potentiation
MAO	Monoamine oxidase
MAOB	Monoamine oxidase type-B
MFB	Medial forebrain bundle
mGluR	metabotropic glutamate receptor
MK-801	(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride
mTORC1	Mammalian target of rapamycin complex 1
NADPH	Nicotinamide-adenine dinucleotide phosphate
NET	Norephinephrine transporter
NMDA	N-methyl-d-aspartate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
ns	Non-significant
PBS	Phosphate buffered saline
PD	Parkinson’s disease
PET	Positron emission tomography
PFA	Paraformaldehyde
RPM	Rotations per minute
p.o.	Per oral
s.c.	Subcutaneous
sGC	Soluble guanylyl cyclase
SN	Substantia nigra
SNpc	substantia nigra pars compacta
SNpr	substantia nigra pars reticulate
STN	Subthalamic nucleus
TH	Tyrosine hydroxylase
Tris-HCL	Tris-(hydroxymethyl)-methylammonium chloride
UPDRS	Unified Parkinson’s Disease Rating Scale
VMAT	Vesicular monoamine transporter
VTA	Ventral tegmental area

Publications

Abstracts

Hirsch, T; Hikima, A; Jackson, MJ; Tayarani-Binazir, K; Fisher, R.; Rose, S; Jenner, P. (2010) Nitric oxide synthase inhibition does not reduce priming for dyskinesia by L-DOPA in a primate model of Parkinson's disease. Poster communication at World Parkinson's Congress II, Glasgow, UK.

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Chapter 1 : General introduction

1.1 Parkinson's disease

The first in-depth description of the disorder now referred to as 'Parkinson's disease' was provided by the English physician, James Parkinson, in 1817 in his essay on the Shaking Palsy (reprinted; Parkinson, 2002). Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's Disease and affects 1-2 % of the population aged over 55 (Di *et al.*, 2007). Both the incidence and prevalence of the disease increase with age with a slightly higher incidence of PD in men compared to women (Elbaz *et al.*, 2002). Current demographic trends predict the number of cases will double by 2050 (Schapira, 2009). Diagnosis of PD is based on clinical manifestation of resting tremor, rigidity, bradykinesia and postural instability (Jankovic, 2008), although these are often accompanied by non-motor symptoms. There is no available treatment to prevent the progress of the disease to date despite ongoing research efforts, and current therapy focuses on symptomatic management.

1.1.1 Aetiology of PD

It is increasingly apparent that there is no single causative factor underlying PD, rather a combination of genetic and environmental factors are likely to be accountable. These may involve multiple interactions between genes, modifying effects by susceptibility alleles, and the influence of environmental agents both indirectly via gene expression and directly on the brain itself (Klein & Schlossmacher, 2007).

PD can be subdivided into sporadic/idiopathic and familial forms, although some genetically determined Parkinson syndromes resemble idiopathic PD but may differ neuropathologically. Most known gene mutations tend to cause juvenile or early onset of the disease (Alves *et al.*, 2008), and many familial identified mutations have been found in apparently sporadic cases (Warner & Schapira, 2003). The genes mutations implicate molecular pathways involved in nigral degeneration specifically including protein aggregation, defective proteasomal degradation, mitochondrial dysfunction and oxidative stress (Litvan *et al.*, 2007a). A number of defined loci are associated with high penetrant autosomal dominant or recessive PD, and causative mutations in specific genes have been identified for 7 of these, although the precise number is subject to some disagreement. These genes include α-synuclein, parkin, UCH-L1 (ubiquitin C-terminal hydrolase-L1), PINK1 (phosphatise and tensin homologue induced putative kinase 1), DJ-1, LRRK2/dardarin (leucine-rich repeat kinase 2) & ATP13A2 (Lee & Liu, 2008; Yang *et al.*, 2009). α-synuclein was the first gene linked to PD and mutations or multiplications can lead to PD pathology specifically including Lewy body formation of which the misfolded and aggregated α-synuclein protein is a major component (Polymeropoulos *et al.*, 1997; Kruger *et al.*, 2002). α-synuclein, parkin and DJ-1 have all been associated with maintenance of physiological dopaminergic function (Lee & Liu, 2008). Additionally, an increased susceptibility to developing PD may be conferred by variants of certain genes such as Glucocerebrosidase (Nichols *et al.*, 2009).

The genetic weighting of the mutations in the idiopathic or sporadic form of PD is small, and accounts for only 5-10 % of the overall PD population. However the strikingly consistent and specific phenotype of

familial and sporadic PD has led some researchers to believe that one common molecular mechanism may underlie PD, although this remains elusive to date (Gandhi & Wood, 2005).

Similarly no specific environmental agent has been identified as causative in PD. Studies have suggested exposure to industrial chemicals in addition to pesticide, herbicide and fungicide products may increase the risk, due to neurotoxic effects. Advantage has been taken of some of these agents in creating animal models of the disease including the use of rotenone and paraquat (Litvan *et al.*, 2007b). Interestingly cigarette smoking has been shown to reduce the risk of developing PD (Elbaz & Tranchant, 2007). Additionally nitric oxide synthase alleles have been shown to eliminate the protective effect of smoking or the risk associated with pesticides in retrospective population studies (Hancock *et al.*, 2008).

1.1.2 Pathology of PD

PD is a chronic progressive neurodegenerative disorder of the extrapyramidal cerebral system classically affecting the basal ganglia (Kadieva & Mutsueva, 2005). The principal biochemical signs of PD are selective degeneration of dopaminergic neurones in the substantia nigra pars compacta (SNc) and the presence of proteinaceous cytoplasmic inclusions, termed Lewy bodies, frequently containing α-synuclein, found in remaining dopaminergic cells (McNaught *et al.*, 2003; Zhang & Goodlett, 2004).

Further widespread pathology is characteristic of PD and other neurotransmitter systems are also affected. Peripherally, decreased tyrosine hydroxylase (TH) activity and decreased dopamine is evident in the adrenal medulla. Centrally, neurotransmitter level changes are detected in the nucleus basalis of Meynert (cholinergic), locus coeruleus (noradrenergic), and Raphe nuclei (serotonergic) in addition to alterations in the glutamatergic and GABAergic pathways (Hodaie *et al.*, 2007). In terms of the evolution of pathological markers of the disease, Braak *et al.* (2003), defined a series of stages of disease progression, evoking some controversy, based on comparing brains obtained at autopsy from diagnosed PD patients, incidental cases without clinical PD symptoms and controls. Typically the dorsal motor nucleus of the medulla oblongata is the first site to be affected by lesions, subsequently extending to the pontine tegmentum and the midbrain, and then the basal prosencephalon and mesocortex. In the final stages the neocortex is subject to significant pathological changes affecting sensory and motor areas. It is only at the stage of loss of approximately 50 % of dopaminergic cells of the substantia nigra (SN) accompanied by depletion of about 80 % of striatal dopamine that the clinical symptoms of PD become apparent (Braak *et al.*, 2003). More recent findings suggest that the motor signs of PD in fact appear when there is only loss of about 30 % of dopaminergic neurons and 50-60 % of striatal dopamine (Cheng *et al.*, 2010).

The degeneration of dopaminergic neurons in the substantia nigra causes a reduction of input to the striatum (the main input gateway connecting the motor cortex to the basal ganglia, and comprising the putamen and caudate nucleus), and in turn this leads to an inbalance in activity of the direct and indirect striatal output pathways (Crossman, 2000). Striatal neurons in the direct pathway project directly from the putamen to both the globus pallidus pars interna (GPi) and the substantia nigra pars reticulata (SNr).

They bear D1 receptors, coexpress the peptides substance P and dynorphin, and provide a direct inhibitory effect on GPi and SNr. Meanwhile striatal neurons in the indirect pathway connect the putamen with the GPi/SNr via synaptic connections in the globus pallidus pars externa (GPe) and subthalamic nucleus (STN). They express D2 receptors and the peptide enkephalin (Obeso *et al.*, 2000b). Dopamine has differential effects on these two receptor types facilitating transmission via D1 receptors and inhibiting transmission via D2 receptors. Dopamine depletion results in increased activity of the indirect pathway and reduced activity of the direct pathway and this alteration has been confirmed by measured changes in preproenkephalin A (neuropeptide co-transmitter utilized at D1 receptors) and preprotachykinin/preproenkephalin B (neuropeptide co-transmitter utilized at D2 receptors) mRNA levels respectively (Gerfen *et al.*, 1990). Within the healthy individual, movement-suppressing pathways and movement-promoting pathways interact to support voluntary movement via physiologically regulated thalamic output to the motor cortex, whilst this balance breaks down in the parkinsonian state resulting in net inhibition of thalamo-cortical output (Obeso *et al.*, 2000b).

1.1.3 Clinical features of PD

Diagnosis of PD is based on clinical manifestation of rest tremor, rigidity, bradykinesia (slowness of movement) and postural instability, although non-motor symptoms including autonomic dysfunction, cognitive/neurobehavioural disorders, and sensory and sleep abnormalities are also common (Jankovic, 2008). Early signs may include a reduced sense of smell (hyposmia) owing to pathologic changes in olfactory structures including the olfactory bulb, although hyposmia is often only noticed retrospectively (Braak *et al.*, 2003).

Some of the most debilitating symptoms of Parkinson's disease are the motor impairments, which are proposed to manifest around 6-8 years after nigral degeneration first occurs (Schapira & Obeso, 2006). The Unified Parkinson's Disease Rating Scale (UPDRS) is the most well established scale to assess the severity of PD symptoms. It evaluates mentation (part I), activities of daily living (ADL; part II) and motor function (part III), and the scores from these subsections are summed to determine the total UPDRS score. Part IV of the UPDRS measures complications of therapy, such as dyskinesia, 'off' periods and sleep disturbance, and is often used in trials of adjunct therapy (Ebersbach *et al.*, 2006; Colosimo *et al.*, 2010).

Fortunately PD has benefited from the development of numerous symptomatic medical therapies, aimed at the dopamine (DA) system, which have proved disease alleviating to some extent, improving daily function, quality of life and survival of those affected (Factor, 2008).

1.2 PD therapy

The predominant therapeutic strategy for addressing the motor symptoms of PD is the use of dopaminergic drugs singularly or in combination, for example Levodopa (L-dopa), dopamine agonists, monoamine oxidase type-B (MAOB) inhibitors, and catechol-O-methyltransferase (COMT) inhibitors (Schapira, 2009). PD may also be treated with non-dopaminergic agents such as cholinergic receptor antagonists. A number of cholinergic agents may be used as monotherapy or as adjuncts, although cognitive and neuropsychiatric side-effects including hallucinations tend to limit their use (Katzenschlager *et al.*, 2003). Anticholinergic agents, such as trihexyphenidyl and benztropine, are the oldest treatments that have been used in PD, and are most effective for treating resting tremor. Anticholinergics are typically reserved for the younger patient (<60 years of age) with predominant tremor and preserved cognitive function (Simuni *et al.*, 2009). Amantadine, an antiviral agent discovered by chance to have antiparkinsonian effects, has demonstrated efficacy for relieving tremor in early PD (Olanow *et al.*, 2001). Amantadine is an older medication with many different actions in the central nervous system, including inhibition of *N*-methyl-D-aspartate receptors, enhancement of dopamine release from presynaptic terminals, and modest anticholinergic activity, although its mode of action is not fully understood (Weintraub *et al.*, 2008). Treatment regimens tend to need to be adjusted as the disease progresses and motor symptoms become more severe, and ultimately all PD patients are prescribed L-dopa.

1.2.1 L-dopa

Since Cotzias et al. first described the beneficial effects of high doses of L-dopa (L-3,4-dihydroxyphenylalanine) on PD patients in 1967, it remains the most effective agent for treatment of motor symptoms in PD and is widely acknowledged as the 'gold-standard' of symptomatic PD therapy (Fahn, 2008). Direct use of dopamine in the treatment of PD is not possible due to its high basicity which impedes penetration through the blood-brain barrier (BBB). Therefore the precursor of dopamine, L-dopa, is strategically employed as a prodrug. It is recognised by L-neutral amino acid transport proteins, crossing the BBB where it is then converted into dopamine within dopaminergic neurons by DOPA decarboxylase (DDC), (Kadieva & Mutsueva, 2005). This conversion can also occur in serotonergic neurones and glia when there is extensive neuronal loss (Schapira, 2009). However L-dopa is rarely administered alone since most of the compound is decarboxylated to dopamine, under the action of peripheral DDC, resulting not only in a low efficacy but also various side-effects including vomiting, nausea and arrhythmia (Lim, 2005). Thus L-dopa is administered in combination with peripheral DDC inhibitors such as carbidopa or benserazide (See **Figure 1-1**).

Other adjunctive treatments can further enhance L-dopa effects as shown in **Figure 1-1**. MAOB inhibitors reduce dopamine metabolism at the synapse and glia thereby enhancing its activity and reuptake. It was concluded that the MAOB inhibitors rasagiline and selegiline are effective as monotherapy in early disease (Goetz *et al.*, 2005), but MAOB inhibitors are also commonly used as an adjunct therapy in more advanced disease to enhance dopamine levels. COMT inhibitors also lessen

dopamine metabolism peripherally as well as centrally leading to prolonged L-dopa effects (Lim, 2005). Entacapone and tolcapone are two selective COMT inhibitors that have been developed for clinical use.

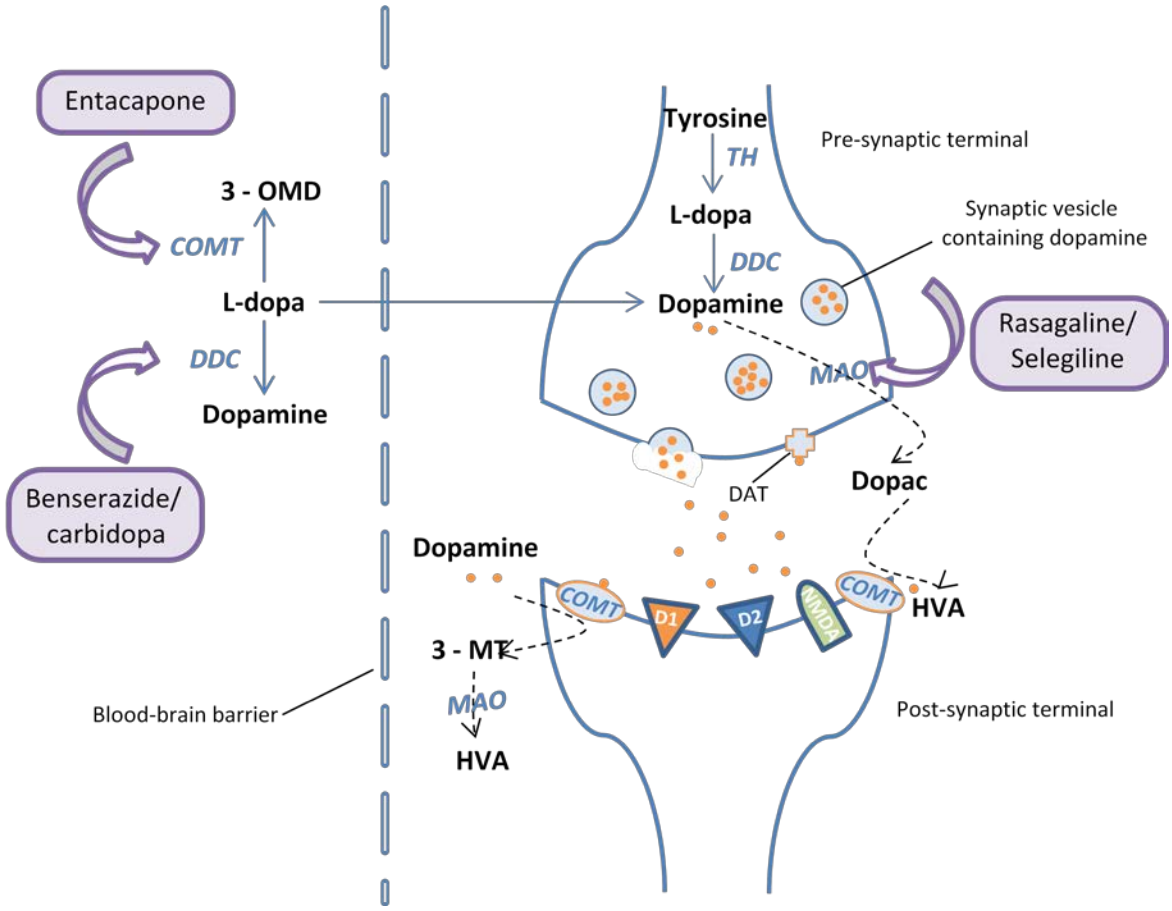

Figure 1-1 Dopamine pharmacology and management of PD. Entacapone is used as a COMT inhibitor and benserazide/carbidopa as a peripheral DDC inhibitor. Rasagiline and selegeline are MAO inhibitors used in the clinic. COMT (catechol-o-methyl transferase), DAT (dopamine active transporter), DDC (Dopadecarboxylase), DOPAC (3,4 dihydrohylphenylacetic acid), D1 & D2 (dopamine receptors), HVA (homovanillic acid), MAO (monoamine oxidase), NMDA (N-methyl-d-aspartate), 3-MT (3-methoxyltyramine) and 3-OMD (3-O-methyldopa).

1.2.2 Dopamine agonists

A range of dopamine agonists are available for use in PD and these fall into the categories of ergot and non-ergot derived. Ergot agonists include bromocriptine, cabergoline, and lisuride, whilst non-ergots include apomorphine, pramipexole, ropinorole and rotigotine. Ergot derived agonists have recently been associated with cardiac valve fibrosis and are less frequently used (Zanettini *et al.*, 2007). Dopamine agonists bypass the degenerating neurons and directly stimulate the intact, although denervated, postsynaptic receptors in the striatum. Dopamine agonists tend to have substantially longer elimination half-lives than L-dopa, allowing for more prolonged stimulation of receptors (Factor, 2008).

The multiplicity of dopamine receptors in the brain offers a range of potential targets, and whilst L-dopa is non-discriminate in its actions, dopamine agonists display a variation in affinity for specific receptor sub-types (see **Table 1-1**). Most currently used dopamine agonists only activate D2 and D3 dopamine receptors and no major advance has been made in producing selective D1 dopamine agonists, a known target for anti-Parkinsonian agents. A number of side-effects of dopamine agonists including hypotension and nausea are related to their action at D2 receptors (Foley *et al.*, 2004). Partial D2 dopamine agonists are being developed as they might treat the motor symptoms of PD while suppressing both psychosis and dyskinesia (Schapira *et al.*, 2006).

Table 1-1 Receptor affinities of dopamine agonists (Jenner, 2002; Foley *et al.*, 2004; Bonuccelli & Pavese, 2007; Factor, 2008).

Dopamine Agonist	Dopamine Receptor Affinity
Bromocriptine	D2>D3>D4>D1
Cabergoline	D2>D3>D4>D1
Lisuride	D2≥D3≥
Apomorphine	D2=D3=D4 > D1
Piribidel	D2=D3>>D1
Pramipexole	D3>D2>D4
Ropinorole	D3>D2>D4
Rotigotone	D3>D4>D2>D1

1.2.3 L-dopa, dopamine agonists and motor complications

In the early stages of PD the response to L-dopa is excellent providing relief of the characteristic motor symptoms experienced. However, motor complications frequently ensue after long-term treatment with L-dopa resulting in “wearing-off” (a decrease in the duration of action of L-dopa) and “on-off phenomenon” (unpredictable switching between mobility benefits of L-dopa and the parkinsonian state) and also, during ‘on’ periods, dyskinesia (uncontrollable involuntary movements) which may become severely disabling in themselves (Duvoisin, 1974). As L-Dopa benefits wear off between doses patients commonly fluctuate amid ‘on’ responses whereby they experience a good antiparkinsonian effect and ‘off’ responses whereby the drug does not adequately treat the parkinsonian features (Obeso *et al.*, 2000a). Motor complications are attributed to a shortening of response, for the same dose of L-dopa, with disease progression from early to later stages of PD (Schapira *et al.*, 2009).

Dopamine agonists (with the exception of apomorphine) have a longer plasma half-life than L-dopa in man and may thus exert a longer period of symptomatic effects. Dyskinesia has been associated with pulsatile stimulation of dopamine receptors and therefore dopamine agonists are often favoured over L-dopa for initial treatment (Lim, 2005; Jenner, 2008b).

Although dopamine agonist monotherapy can effectively control motor symptoms for several years, most patients inevitably require L-dopa supplementation at some stage during the disease. There are conflicting results of holding off L-dopa in reducing motor complications and consideration of the adverse events reported is important (Schapira *et al.*, 2009).

COMT catalyses the conversion of dopamine into 3-O-methyldopamine, and of L-dopa into 3-O-methyldopa (Jorga *et al.*, 1998). The use of COMT inhibitors in conjunction with L-dopa can therefore increase the quantity of dopamine reaching the striatum and reduce the dose of L-dopa required. By increasing the half life of L-dopa the duration of effect is also prolonged and pulsatility of dopamine receptor activity reduced (Nutt *et al.*, 1994). The addition of COMT inhibitors such as entacapone or tolcapone to dopaminergic medication has been shown to increase the amount of time motor disability is beneficially reversed ('on-time') by 1.3-1.8 h per day in PD patients, although with no accompanying reduction in dyskinesia (Group, 1997; Deane *et al.*, 2004). COMT inhibitors may therefore be most useful in patients showing wearing-off effects and may require reductions in L-dopa dose.

Whether continuous dopaminergic stimulation explains all of the differences between L-dopa and dopamine agonists is questionable, and there might instead be fundamental differences between the actions of these agents. L-Dopa acts on both D1 and D2 receptor families, whereas dopamine agonists tend to be more specific to D2-type receptors. In PD patients D1 agonists induce dyskinesia to the same extent as L-dopa whereas D2/D3 agonists induce little dyskinesia as monotherapy (Rascol *et al.*, 2006). There is also evidence suggesting L-dopa affects multiple pharmacological targets including nonadrenaline and 5-hydroxytryptamine (5-HT; serotonin) receptors and might act as a neuromodulator in its own right although dopamine agonists may also interact with other neurotransmitter pathways (Mercuri & Bernardi, 2005; Schapira *et al.*, 2006). Nigrostriatal neurons also show increased responsiveness to L-dopa as compared to the dopamine agonist ropinirole in a rodent model of PD (Carta *et al.*, 2008a), although the exact reasons for differences between these agents remains uncertain.

1.3 Dyskinesia

The term dyskinesia refers to the abnormal involuntary movements that manifest clinically following L-dopa treatment in PD patients. Although they are seen most commonly in conjunction with L-dopa their expression is also seen after long-term dopamine agonist medication. Indeed up to 80 % of patients

receiving dopaminergic treatment show dyskinesia within 5 years (Rascol *et al.*, 2000). Dyskinesia may take the form of chorea (rapid dancing movements), dystonia (persistent muscle contraction) or athetosis (writhing movements) (Fahn, 2000). Based on the temporal relationship with drug dosing these can be further classified as peak-dose or diphasic dyskinesias and off-dose dystonias (Jankovic, 2005; Thanvi *et al.*, 2007).

1.3.1 Risk factors

Evidence shows that the onset and severity of dyskinesia is associated with several factors including the extent of nigral dopaminergic cell loss, the type of drug administered and also the mode of drug delivery. Following initiation of L-dopa, the appearance of dyskinesia occurs earlier in more severely affected patients, who accordingly suffer from a greater loss of dopaminergic neurons (Ahlskog & Muenter, 2001). L-dopa does not induce dyskinesia in healthy people or animals suggesting nigral denervation is a necessary factor (Boyce *et al.*, 1990). There are several exceptions where high doses of L-dopa have caused dyskinesia in naive primates (Pearce *et al.*, 2001) implying that the intact basal ganglia may be able to generate dyskinesia under extreme conditions, whilst nigral denervation appears to lower the threshold for its induction.

The age of onset of PD is also linked with the extent of dyskinesia which manifests over 5 years of treatment (Kumar *et al.*, 2005). With PD onset after 70 years of age, the incidence was 16 %, whereas with onset from 40 to 59 years of age, the incidence raised to 50 %. This difference in dyskinesia development may be due to age-related physiological changes within the dopaminergic system. Early monotherapy employing long-acting dopamine agonists is associated with a lower incidence of dyskinesia in the clinic than occurs with L-dopa treatment (Shannon *et al.*, 1997; Rascol *et al.*, 2000; Inzelberg *et al.*, 2003). Whilst the use of dopamine agonist drugs as early monotherapy reduces the risk of dyskinesia induction when L-dopa therapy is inevitably introduced with disease progression, it appears at the same rate as in those patients treated initially with L-dopa (Rascol *et al.*, 2006). Higher doses of L-dopa (>400 mg/day) also seem to increase the incidence of dyskinesia, and motor complications tend to be more common in woman than men, as well as in patients with a body weight under 75 kg (Stocchi *et al.*, 2010). Longer acting dopamine agonists induce lower levels of dyskinesia and the occurence of dyskinesia is lower with dopamine agonists compared with L-dopa (Rascol *et al.*, 2000; Oertel *et al.*, 2006). This variation is possibly owing to the relatively short plasma half life of L-dopa of 90 min (Nutt, 2008). This causes pulsatility in receptor stimulation and accordingly it has been shown that repeated administration of short acting dopamine agonists produces more dyskinesia than continuous infusion of the same drug (Bibbiani *et al.*, 2005a). Controlled-release formulations aim to overcome this problem and duodenal L-dopa infusion for example can reduce dyskinesia in some patients (Antonini *et al.*, 2007; Devos, 2009).

1.3.2 Pathophysiology of dyskinesia

'Priming' refers to the induction processes by which the brain becomes sensitised, whereby each administration of dopaminergic therapy modifies the response to subsequent dopaminergic treatment

(Brotchie, 2005). Priming may take place from first administration of dopaminergic medication and can continue with repeated treatment until dyskinesia reaches stable levels. There is a difference between the induction of dyskinesia i.e. priming which occurs with both dopamine agonists and L-dopa and the expression which may be lower when dopamine agonists are used as compared to L-dopa (Jackson *et al.*, 2007). It is the mechanisms underlying priming, and also the consequential expression and maintenance of dyskinesia that are still not fully understood. However significant progress toward this end has occurred in recent years especially with regard to molecular changes associated with dyskinesia (Jenner, 2008b).

At a circuitry level an array of changes are thought to take place within the basal ganglia which differentiate it from the conditions present in the healthy and parkinsonian state. According to the schematic model of the basal ganglia (see **Figure 1-2**), changes opposite to those proposed to underlie the parkinsonian state (as described in section 1.1.2), would result in reduced inhibition of thalamo-cortical neurons and output of motor areas synonymous with dyskinesia i.e. hypoactivity of the indirect pathway and hyperactivity of the direct pathway. These changes are supported by electrophysiological studies showing increased neuronal activity in the GPe and reduced neuronal firing in the GPi during dyskinesia (Filion & Tremblay, 1991; Lozano *et al.*, 2000). However further studies bring to light the shortcomingsof this model whereby stereotaxic lesioning of the GPi reduces dyskinesia rather than increases it as the model would predict (Parkin et al., 2002). Altered firing patterns within the basal-ganglia–thalamocortical loops may be as important as overall changes in firing rates to the emergence of dyskinesia (Boraud et al., 2001). Whilst the schematic model forms a good basis for considering the phenomenon of dyskinesia, examining more specific changes may provide a more comprehensive understanding.

Presynaptic changes (resulting from reduced dopaminergic fibres combined with chronic L-dopa administration) have been associated with altered efficiency of the dopamine transporter (DAT) in dyskinesia, as well as changes in cellular compartments regulating influx and handling of exogenous L-dopa including serotonergic projections from midbrain raphe nuclei (Tanaka *et al.*, 1999; Troiano *et al.*, 2009). Striatal expression levels of prodynorphin (pre-proenkephalin B) mRNA and Fos-B/ΔFos-B related transcription factors positively correlate with severity of L-Dopa - induced dyskinesia (Cenci *et al.*, 1998; Lundblad *et al.*, 2004). Furthermore these two markers are persistently upregulated for several weeks following L-Dopa treatment (Andersson *et al.*, 2003). This more long-term biochemical alteration may signify plasticity changes related to dyskinesia induction. Overactive signaling through D1 receptors has been linked to these changes (Cenci & Lundblad, 2006).

Various postsynaptic changes in D1 receptors in association with dyskinesia have been reported, meanwhile D2 receptors seem only moderately affected in dyskinesia, whilst there is some evidence for

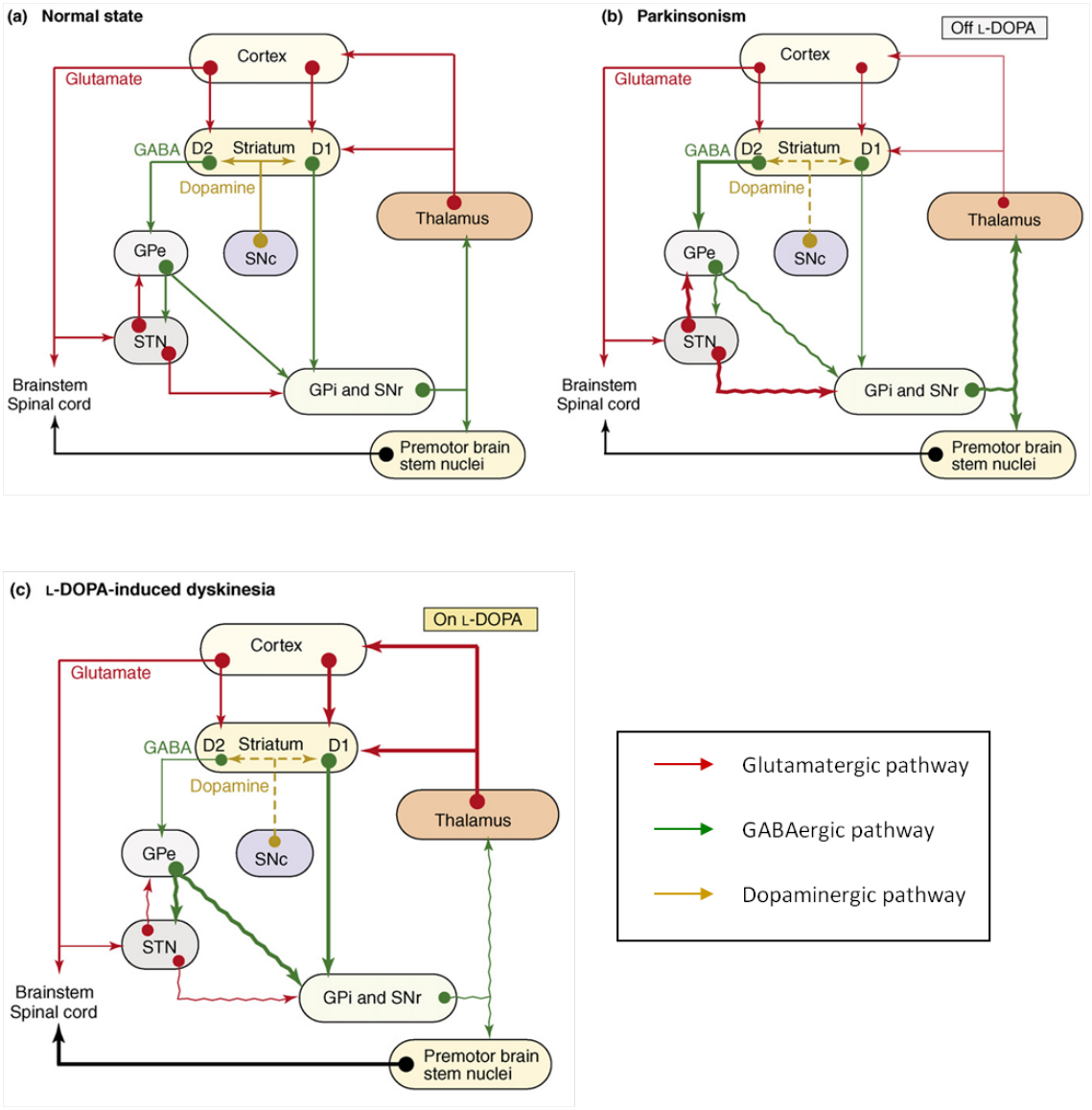

Figure 1-2 The basal-ganglia–thalamocortical circuits under normal conditions **(a)**, in the untreated parkinsonian state **(b),** and in L-dopa-induced dyskinesia **(c)**. (black lines indicate chemically composite pathways). In b and c, changes in the average activity rate of specific projection pathways are shown as thickening (increased activity) or thinning (decreased activity) of the corresponding lines compared with the normal state. Alterations in firing patterns are represented by sinuous lines. The striatum and the STN provide the input layer for incoming cortical information to the basal ganglia. The GPi and SNr provide the output layer communicating with the rest of the brain. These structures exert a strong inhibitory control on their projection targets in the thalamus and the brainstem and this tonic inhibitory input must be released to enable normal movements to occur. The striatum exerts opposite influences on the GPi and SNr via two distinct classes of efferent neurons, the D1-receptor-rich 'direct pathway', which is positively modulated by dopamine, and the D2-receptor-rich 'indirect pathway', which is negatively modulated by dopamine. The loss of dopamine in PD (b) causes an imbalance in the activity of the two striatofugal pathways and their corresponding cortical inputs. The basal-ganglia-thalamocortical circuit during the expression of dyskinesia (c), the imbalance assumes an opposite sign. (GPe, external segment of the globus pallidus; GPi, internal segment of the globus pallidus; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus (Cenci, 2007)).

increased D3 receptor expression within the dorsal striatum (Bordet *et al.*, 1997; Konradi *et al.*, 2004; Nagai *et al.*, 2007; Darmopil *et al.*, 2009). Sensitisation of D1 receptors and also their increased density have been found in the striatum of MPTP-treated primates exhibiting dyskinesia (Aubert *et al.*, 2005; Guigoni *et al.*, 2005). These are thought to initiate further downstream changes in signaling pathways involving cAMP and ERK 1/2 possibly due to an increased coupling of D1 receptors to their transducing G proteins (Gerfen *et al.*, 2002; Santini *et al.*, 2008). Sensitisation of D1 receptors results in enhanced activation of cAMP-dependent protein kinase A which phosphorylates downstream proteins including DARPP-32. The striatum-specific signaling protein DARPP-32 is found to show a persistant increase in phosphorylation at Thr-34 in dyskinetic animals compared to non-dyskinetic and drug naïve animals (Picconi *et al.*, 2003; Santini *et al.*, 2007). Recently down-regulation of CalDAG-GEFI and up-regulation of CalDAG-GEFII mRNA's and proteins, both regulators of ERK signaling, have been found to be closely correlated with severity of dyskinesia (Crittenden *et al.*, 2009). It therefore seems D1 receptor related changes are important in terms of dopaminergic contributions to dyskinesia.

Overactivity of glutamatergic systems in the basal ganglia have also been implicated in dyskinesia, and increased sensitivity of N-methyl-D-aspartate (NMDA) receptors may contribute to the longer term activity dependent alterations in neuronal responses (Chase *et al.*, 2003). In fact a recent PET study in PD patients found abnormally enhanced glutamatergic transmission in motor areas following L-dopa administration in dyskinetic patients (Ahmed *et al.*, 2011). NMDA and dopamine receptors are expressed in close proximity along the dendrites of medium spiny neurons and mechanisms of interaction following D1 receptor activation such as protein kinase A dependent phosphorylation of NMDA subunits and increased expression of the NMDA receptor subunit 2B (NR2B) have been evident in dyskinesia (Leveque *et al.*, 2000; Hurley *et al.*, 2005; Hallett *et al.*, 2006). NMDA receptors showing altered subunit composition form complexes with D1 receptors and these are trafficked abnormally between the postsynaptic membrane and intracellular compartments or extrasynaptic membrane sites in animals showing dyskinesia (Gardoni *et al.*, 2006; Fiorentini *et al.*, 2008). Furthermore NMDA receptor antagonists reduce dyskinesia in primate and rodent models of PD (Blanchet *et al.*, 1999; Lundblad *et al.*, 2002; Nash & Brotchie, 2002; Morissette *et al.*, 2006).

Due to their gradual development, persistent character and apparent dependence on glutamate-receptor mechanisms, dyskinesias that arise in PD patients treated with L-dopa have been compared to an aberrant form of motor learning and synaptic plasticity (Calabresi *et al.*, 2000; Chase & Oh, 2000). Indeed there is a loss of ability for "depotentiation" in the 6OHDA rat model of PD seen only in animals expressing dyskinesia (Picconi *et al.*, 2003; Belujon *et al.*, 2010). Additionally, endocannibanoids, opioids, serotonin and noradrenaline are all proposed to influence abnormal synaptic transmission underlying dyskinesia (Brotchie, 2005). Considering its close link to glutamate and D1 receptor activity, nitric oxide is another candidate that has been advocated for involvement in aberrant synaptic plasticity mechanisms within the striatum (Calabresi *et al.*, 1999; West & Grace, 2000; Sammut *et al.*, 2006). Much of our knowledge concerning pathophysiology and also therapeutics for dyskinesia has been

achieved through investigations in animal models of PD (Bove et al., 2005; Lane & Dunnet 2008; Duty & Jenner 2011).

1.3.3 Experimental animal models of PD and dyskinesia

Ideally animal models of disease should demonstrate all the relevant pathology and symptoms present in humans, however this is rarely possible. Instead in modelling complex disorders such as PD it is important to employ a model which best displays the features associated with a particular line of investigation. The recent developments in genetics have led to the generation of transgenic and knock-out mouse models expressing mutant proteins involved in familial PD (Goldberg *et al.*, 2003; Wakamatsu *et al.*, 2008). Whilst useful in studying aetiology they tend to be relatively poor at predicting responses to therapeutic agents (Fleming *et al.*, 2005). The most extensively employed models to investigate dyskinesia in Parkinson's disease are the toxin based 6-hydroxydopamine (6-OHDA)-lesioned rat and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated primate which both reliably reproduce the common motor complications seen in man (Jenner, 2008a; Cenci & Ohlin, 2009). Indeed the combined use of rodent and primate models affords target identification and preclinical validation of therapeutic efficacy in PD (Bezard & Przedborski, 2011).

1.3.3.1 The 6-OHDA-lesioned rat

The neurotoxin 6-OHDA has become one of the most commonly used toxins for modelling PD in rodents since Ungerstedt first established a bilateral working model in 1968. This was later modified to a unilateral model owing to a high mortality rate where 6-OHDA had been administered into both sides of the brain (Ungerstedt & Arbuthnott, 1970). As the toxin does not efficiently cross the blood-brain barrier a direct injection into the brain is necessary. 6-OHDA principally exerts its effects on catecholaminergic pathways and its mechanism of action is related to combined effects of reactive oxygen species (ROS) and quinines (Przedborski & Ischiropoulos, 2005). The toxin is taken up by dopaminergic neurons via the DAT transporter and norepinephrine neurones by the NET transporter. Selective degeneration of dopaminergic neurons can be achieved by administration of the NET inhibitor, despiramine prior to 6-OHDA treatment.

By using different injection sites and doses of 6-OHDA varying extents of neurodegeneration can be reproduced according to the experimental paradigm from inspecting molecular events to modelling motor symptoms of PD (Simola *et al.*, 2007). Lesions of the medial forebrain bundle (MFB) which projects dopaminergic neurones from the SNc to the striatum causes dopaminergic neurons to degenerate within the first 24 h and within 3–4 days almost complete striatal cell death is evident (Deumens *et al.*, 2002; Bove *et al.*, 2005).

Pre-clinically, potential drug treatments for the reduction of dyskinesia are commonly tested in the unilateral 6-OHDA-lesioned rat primed to display abnormal involuntary movements (AIMs) by chronic dopaminergic therapy over several weeks (Cenci *et al.*, 1998). The AIMs model is a useful behavioural

model of motor-abnormalities accompanying long-term dopaminergic medication in PD, with distinct advantage over the traditional rotational model, as the animals display abnormal movements which are both choreic (e.g. limb) and dystonic (e.g. axial) in nature (Marin *et al.*, 2006; Cenci & Ohlin, 2009). In dopaminergic-treated 6-OHDA-lesioned rats there is some divergence on the interpretation of rotational behaviour, which is considered to mimic anti-parkinsonian activity and hence beneficial drug activity, versus the problematic phenomena of dyskinesia (Marin *et al.*, 2006). Indeed, L-dopa induced rat AIMs functionally mimic the peak-dose dyskinesia seen in PD patients and are accompanied by molecular changes similar to those evident in primate models of PD displaying dyskinesia (Carta *et al.*, 2006). Importantly drugs with proven anti-dyskinetic efficacy in the clinic (e.g. amantadine) also improve motor complications in the rat model (Lundblad *et al.*, 2002).

1.3.3.2 The MPTP-treated primate

The inadvertent discovery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) following human recreational drug use revolutionised pharmacological research of PD. 'Synthetic heroin' was injected by drug addicts in the 1980's containing traces of the toxic meperidine metabolite MPTP, leading to the rapid onset of a marked and enduring parkinsonism (Langston *et al.*, 1983). This incident paved the way for development of the MPTP-treated primate model of PD where formerly only electrolytic or radiofrequency lesioning of the basal ganglia had been used in this species (Poirier *et al.*, 1975).

MPTP readily crosses the blood-brain barrier where it is oxidised by MAOB within glial cells to 1-methyl-4-phenylpyridinium (MPP+) (Smeyne & Jackson-Lewis, 2005) . MPP+ is selectively taken up by the dopamine uptake system where it inhibits complex 1 of the mitochondrial respiratory chain resulting in cytotoxic free radical formation (Nicklas *et al.*, 1987; Singer *et al.*, 1988) . Humans and non-human primates are particularly susceptible to MPTP-induced toxicity but rodents tend to be resistant to its effects with the exception of certain strains of mice. The lack of toxic effects in most rodent species may be attributed to the relatively rapid clearance of MPP+ (Johannessen *et al.*, 1985). Systemic administration of MPTP over several days leads to an onset of the major motor symptoms of PD in primates including bradykinesia, rigidity and postural abnormalities, closely mimicking clinical aspects of the disease (Jenner *et al.*, 1984; Crossman *et al.*, 1987). The behavioiural symptoms have been characterised in primates including the cynamalogues (*Macaca fascicularis*), common marmosets (*Callithrix jacchus*) and squirrel monkeys (*Saimiri sciureus*), although New World are favoured over Old World primates in PD research owing to handling, housing and ethical issues (Brotchie & Fox, 1999).

Most neuropathological studies in primates have described highly selective damage to dopaminergic neurones of the substantia nigra accompanied by a 95-99 % depletion of dopamine in the caudate nucleus and putamen (Bloem *et al.*, 1990). Thus it is a model of only selective nigrostriatal degeneration with little effect in other regions such as the VTA, although cell loss in the hypothalamus and the locus coeruleus have been reported (Crossman *et al.*, 1985; Gibb *et al.*, 1986). Other regions commonly affected in PD, including the raphe nuclei, substantia innominata, and dorsal motor nucleus of vagus appear undisturbed (Garvey *et al.*, 1986). The loss of dopaminergic neurones is not progressive as in

humans and Lewy bodies do not manifest although accumulations of α-synuclein may occur (Kowall *et al.*, 2000; Halliday *et al.*, 2009).

Dopaminergic drugs typically used for the treatment of PD including L-dopa and ropinirole are able to reverse the MPTP-induced motor symptoms seen in primates, and animals can be rated on motor disability scales based on clinical assessment (Pearce *et al.*, 1998; Imbert *et al.*, 2000). Furthermore the chronic administration of L-dopa or dopamine agonists reliably reproduces dyskinesia consisting of chorea, dystonia and athetosis and in addition to peak-dose dyskinesia both 'end-of dose deterioration,' the 'on–off' phenomena and rebound worsening have been reported also closely resembling the clinical scenario (Clarke *et al.*, 1987; Pearce *et al.*, 1995; Langston *et al.*, 2000; Kuoppamaki *et al.*, 2002). The MPTP-primate model has high predictive validity for translation of dopaminergic drugs treating PD into the clinic (Fox *et al.*, 2006). All of the dopaminergic drugs used for treatment of PD have proven effective in the MPTP-model including apomorphine, bromocriptine, cabergoline, pergolide, pramipexole and ropinirole, in addition to antimuscarinic agents tested such as trihexyphenidyl (Close *et al.*, 1990; Fukuzaki *et al.*, 2000a; Jenner, 2003; 2008a). Similarily the anti-dyskinetic agent amantadine and alterations in dopaminergic dosing regimens have proved effective in reducing motor complications in the primate model akin to the human condition (Bedard *et al.*, 1986; Blanchet *et al.*, 1998a; Smith *et al.*, 2005).

1.3.4 Therapeutic options addressing dyskinesia

Aside from adjustments made to dopaminergic medication in an attempt to slow down the induction of dyskinesia including the addition of COMT inhibitors and/or MAOB inhibitors to extend the duration of activity of L-Dopa (and thus reduce pulsatility), and combining optimum dose of L-dopa and dopamine agonist for maximum antiparkinsonian effect with minimum dyskinesia (Rascol *et al.*, 2002a; Pahwa *et al.*, 2006), relatively few options are available in the later stages of PD. Once dyskinesia manifests, some evidence suggests its expression tends to be lower with dopamine agonists as opposed to L-dopa, as demonstrated in PD patients and MPTP-treated primates (Kapoon *et al.*, 1989; Facca & Sanchez-Ramos, 1996; Hadj Tahar *et al.*, 2000; Jackson *et al.*, 2007). Others report that once dyskinesia has appeared it is equally evoked by L-dopa and dopamine agonists and switching to dopamine agonist treatment rarely works sustainably, and typically any reduction seen in dyskinesias is at the expense of less satisfactory control of parkinsonian symptoms (Encarnacion & Hauser, 2008; Stocchi *et al.*, 2008).

In severe cases, patients may be switched to high dose dopamine agonist monotherapy in an attempt to alleviate the dyskinesia but this approach is not tolerated by the majority of patients because of psychiatric side effects (Stocchi *et al.*, 2008). Continuous subcutaneous infusion of apomorphine or enteral L-dopa infusions can also reduce dyskinesia intensity in some cases (Katzenschlager *et al.*, 2005). In particular duodenal L-dopa infusion (Duodopa) can reduce dyskinesia especially in advanced PD patients, although practical implications can limit application (Antonini *et al.*, 2007). Where medical

therapy does not provide sufficient relief surgical intervention is an option and pallidotomy, or deep brain stimulation of STN and GPi, may be highly effective (Delong & Wichmann, 2007).

Of symptomatic therapy options, the non-competitive NMDA antagonist amantadine is the only effective medication available for reducing the severity of dyskinesia without worsening of parkinsonian symptoms (Snow *et al.*, 2000; da Silva-Junior *et al.*, 2005; Wolf *et al.*, 2010). Interestingly the beneficial effect of amantadine lends further support to a fundamental role for glutamatergic pathways in dyskinesia. However there is limited tolerance of amantadine due to side-effects including psychosis and oedema, and there is some evidence showing only a small proportion of PD patients respond well and accompanying benefit may only last for several months (Crosby *et al.*, 2003; Thomas *et al.*, 2004).

Given the association of increasing severity of dyskinesia with reductions in quality of life measures and significant health care costs it is desirable to find broadly effective medication to suppress if not prevent the occurrence of dyskinesias (Pechevis *et al.*, 2005; Buck & Ferger, 2010). To this end many different agents have been proposed to date especially those centred upon non-dopaminergic pathways implicated in PD including serotonergic, adrenergic, cholinergic & peptidergic systems, as well as further glutamatergic drugs beyond amantadine, but few have proved satisfactory in the clinic (Stacy & Galbreath, 2008; Buck & Ferger, 2010). Neuronal nitric oxide synthase (nNOS) inhibitors, targeting the neurotransmitter nitric oxide, could provide a new angle for addressing unmet therapeutic need for symptomatic treatment of dyskinesia (Jenner, 2008b).

1.4 NO, nNOS and nNOS inhibitors

NO is a ubiquitous signalling molecule readily able to diffuse across cell membranes and involved in a wide range of biological responses from gene induction to immune stimulation and smooth muscle relaxation (Mayer & Hemmens, 1997; Patel *et al.*, 1999). NO was initially described in 1987 as the 'endothelial derived relaxation factor' (Palmer *et al.*, 1987), and much has been learnt about its physiological functions since then. NO acts as a neurotransmitter and/or neuromodulator in both central and peripheral nervous systems by cyclic guanosine monophosphate (cGMP)-dependent mechanisms (Garthwaite, 1991; Prast & Philippu, 2001). Of particular relevance here is its role in mediating excitatory glutamatergic neurotransmission in brain regions including the striatum, where the activation of NMDA receptors stimulates NO release (which in turn modulates glutamate discharge), (Garthwaite *et al.*, 1988; Trabace & Kendrick, 2000).

1.4.1 Synthesis of NO

Endogenous NO is not stored but generated on demand from L-arginine which is oxidised to L-citrulline in a calmodulin dependent reaction catalysed by nitric oxide synthase (NOS) enzymes (Marletta *et al.*, 1998) (see **Figure 1-3**). Three major NOS isoforms exist named according to the conditions in which they

were first described; endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS), (Alderton *et al.*, 2001). Additionally mtNOS (mitochondrial NOS) has been recognised and is thought to be an isoform of nNOS with post-translational modifications present in the inner mitochondrial membrane (Elfering *et al.*, 2002). eNOS and nNOS are constitutively expressed and calcium dependent, whereas iNOS is expressed in response to immunological or inflammatory stimulation and its activity does not depend on intracellular calcium levels. All the NOS enzymes share 50-60 % sequence homology (Lamas S., 1992), and require molecular oxygen and NADPH as co-substrates. Each NOS isoform possesses four prosthetic groups: flavin adenine dinucleotide (FAD) and flavin adenine mononucleotide (FMN) form the oxygen domain, whilst iron protoporphyrin IX (heme) and tetrahydrobiopterin (BH_4) form the reductase domain (Alderton *et al.*, 2001). NOS's are functional only as dimers which it is believed activates the enzyme by sequestering iron, generating high-affinity binding sites for arginine and BH_4 and allowing electron transfer from the reductase to the oxygenase domain (Vallance & Leiper, 2002). A zinc centre is located below the monomer interface.

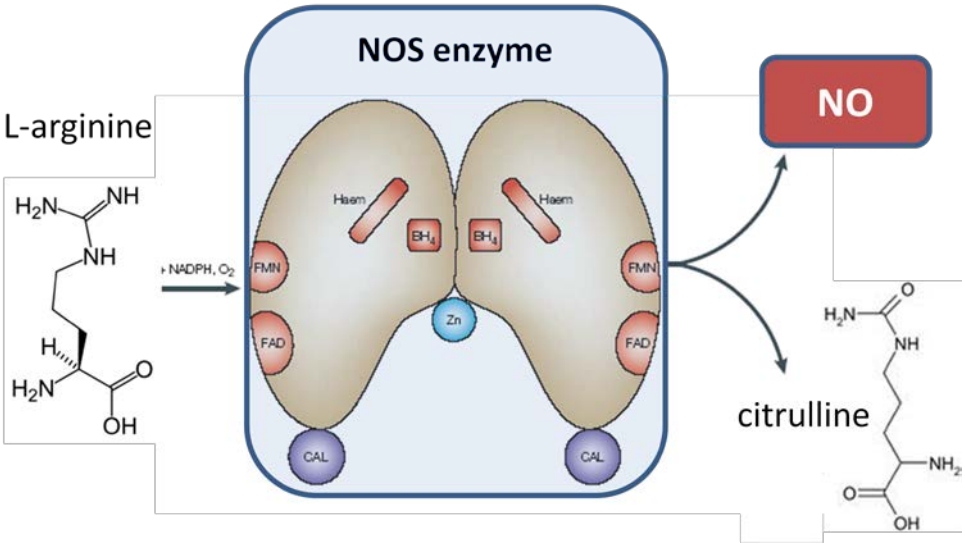

Figure 1-2 The NOS pathway. For enzymatic activity, nitric oxide synthase (NOS) enzymes must dimerize and bind the cofactors tetrahydrobiopterin (BH4), haem, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). On binding calmodulin (CAL), the active enzyme catalyses the oxidation of L-arginine to citrulline and nitric oxide (NO) and requires molecular oxygen and NADPH as co-substrates. Each NOS dimer coordinates a single zinc (Zn) atom. (Adapted from Vallance & Leiper, 2002).

1.4.1.1 iNOS

Under normal physiological conditions iNOS is not expressed in healthy cells. Instead it is induced in response to various inflammatory stimuli such as tumour necrosis factor (TNF), interferon-gamma, or lipopolysaccharide (MacMicking *et al.*, 1997). Although first identified in macrophages iNOS can be rapidly transcriptionally induced in multiple cell types including vascular endothelial and smooth muscle cells where it is functionally located to the cytosol composed of 130 kDa subunits (Stuehr *et al.*, 1991; Charles *et al.*, 1993). iNOS expression is important for host defence against certain protozoa, bacteria,

fungi & viruses as well as skin wound healing and healing of intestinal mucosa (Bogdan, 2001). Despite this favourable role, the large amounts of NO produced by iNOS can contribute to the tissue damage seen in a multitude of disorders such as autoimmune and inflammatory diseases (Vane *et al.*, 1994). It has also been linked to the pathological cascade of events seen in neurodegenerative diseases including Alzheimer's and indeed Parkinson's disease (Knott *et al.*, 2000; Heneka & Feinstein, 2001).

1.4.1.2 eNOS

eNOS is constitutively expressed in endothelial cells of the entire vasculature including vessels of the brain and heart (Wu, 2002). It is a 135 kDa protein and the only NOS subtype that is membrane-associated (Lamas S., 1992). eNOS is localised to the caveolae which are microdomains of the plasmalemmal membrane that are implicated in a variety of cellular functions including signal transduction events (Garcia-Cardena *et al.*, 1996). Several chemical signals in blood vessels activate eNOS including acetylcholine (acting via muscarinic M3 receptors), bradykinin, and vascular endothelial growth factor stimulating an increase in intracellular Ca^{2+} (Benarroch, 2011). Meanwhile shear stress elicits eNOS independent from Ca^{2+} changes. NO generation from the endothelium is important for maintenance of the vasculature in a relaxed state and inhibition of the adhesion of platelets and white blood cells, in addition to suppression of the replication of smooth-muscle cells (Napoli & Ignarro, 2001). The vasoprotective properties conferred by eNOS are confirmed by studies in animal models showing vasoconstriction, hypertension, enhanced platelet activation and increased atherogenesis following pharmacological NOS inhibition (Vallance & Leiper, 2002).

1.4.1.3 nNOS

nNOS is expressed in populations of developing and mature neurons, and to a lesser extent in some cerebral vessels and glial cells, and is predominantly located in the cytosol distributed in a patch-like form (Bredt *et al.*, 1990; Salter *et al.*, 1991). In the CNS nNOS is associated with neurotransmission, synaptic plasticity, neurogenesis, regulation of the sleep-wake cycle and body temperature in addition to neuroprotective and neurotoxic effects (Calabrese et al., 2007). High levels of nNOS are found in the mammalian cerebellum, olfactory bulb, striatum and hippocampus (Bredt *et al.*, 1991; Egberongbe *et al.*, 1994). Innervation of the basal ganglia involves nNOS; in the subthalamic nucleus (STN) more than 95 % of neurons are NOS mRNA positive whilst in the striatum 1.5 to 2 % of neurons are NOS positive, although levels of expression per neuron are considerably higher in the striatum compared to the STN (Nisbet *et al.*, 1994; Blum-Degen *et al.*, 1999).

Neuronal NOS of striatal medium aspiny interneurones colocalises with the peptidergic modulators somatostatin and neuropeptide Y in addition to the neurotransmitter GABA, and all nNOS interneurones co-express nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d), (Dawson *et al.*, 1991; Kubota *et al.*, 1993; Figueredo-Cardenas *et al.*, 1996). nNOS has a PDZ (post-synaptic density protein, discs-large, ZO-1) domain through which it can interact with other proteins, and is thought to enable

linking of signal transduction pathways in multiple complexes (Chanrion *et al.*, 2007). Four splice variants of nNOS have recently been identied (nNOSb, nNOSg, nNOSm and nNOS-2) and these appear to exhibit distinct cellular and tissue localisation, although in the brain the full length 160 kDa nNOSα is predominant (Alderton *et al.*, 2001). nNOS can be located either pre- or post-synaptically and is particularly implicated in neural signalling, neurotoxicity, synaptic plasticity and modulation of behavioural pathways such as learning or expression of pain (Esplugues, 2002).

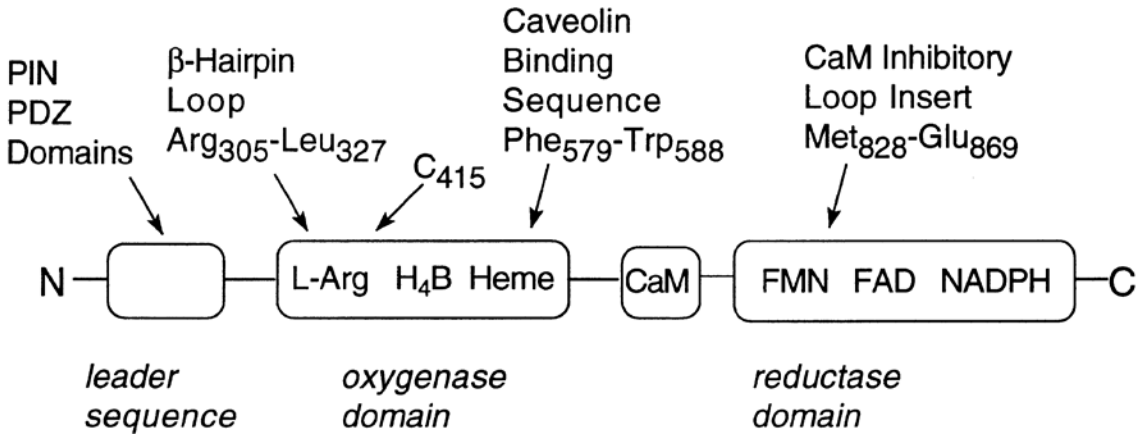

Figure 1-3 Domain arrangement in rat nNOS (Stuehr, 1999).

1.4.2 NO, the basal ganglia and synaptic plasticity

In the CNS nitric oxide (NO) is released following stimulation of nNOS in response to NMDA receptor activation (Garthwaite *et al.*, 1989). Within the striatum, dopamine plays an important modulatory role in association with glutamate in regulating long term-plasticity (LTP) and long-term depression (LTD), which are both activity-dependent forms of modified transmission efficacy occurring at synapses (West et al., 2002). These changes are able to alter neurotransmission efficacy within the basal ganglia (see **Figure 1-5**).

Whilst most studies indicate that NO is derived from nNOS at striatal neurons, work involving mice lacking the eNOS gene suggests that NO released from the vasculature may also be capable of modulating corticostriatal transmission (Doreulee *et al.*, 2003). The presence of NOS immunoreactivity in striatal axon terminals supports the hypothesis that NO can be released by synaptic terminals (Guevara-Guzman *et al.*, 1994). There is also evidence for convergence of such inputs from the cortex and substantia nigra at individual spines of striatal medium spiny projection neurons in the rat (Smith & Bolam, 1990). This localisation may provide a specific site for the interaction of nigrostriatal dopaminergic input with input from the cerebral cortex, and NOS terminals synapsing with spines may allow for direct regulation of the activity of striatal spiny projection neurones in addition to cortical and thalamic inputs (Morello *et al.*, 1997; Hidaka & Totterdell, 2001).

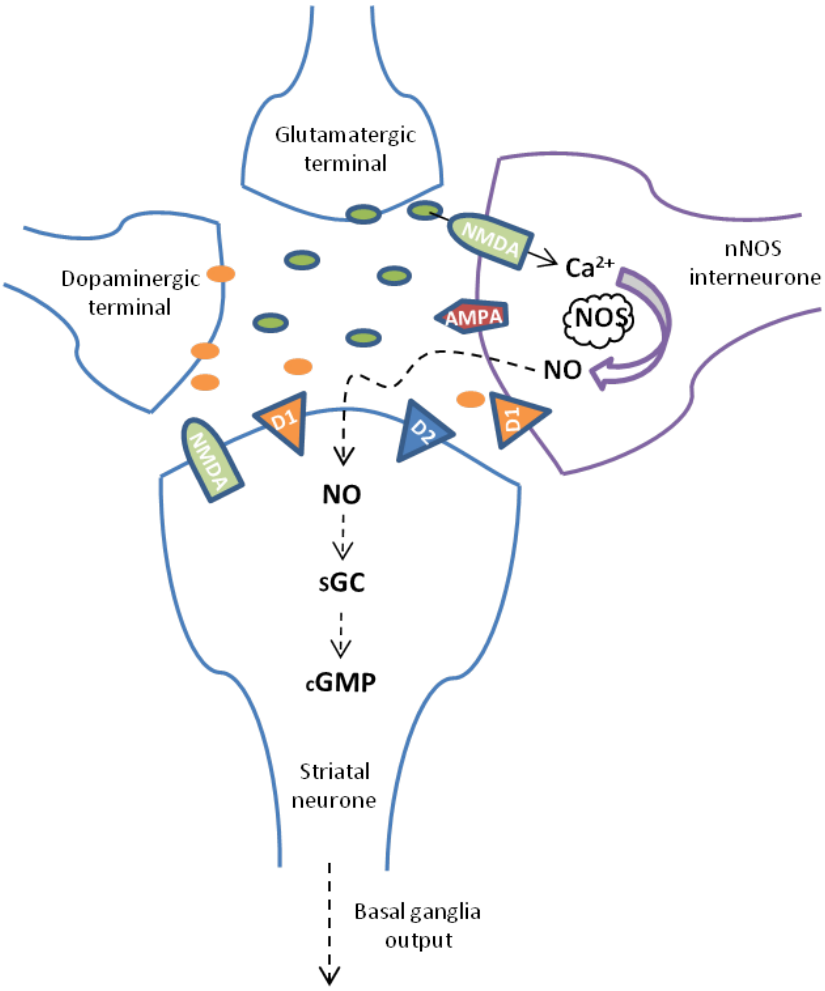

Figure 1-4 Schematic diagram of convergent action of dopaminergic, glutamatergic and nNOS transmission on medium spiny striatal neurones. NO released from nNOS interneurones is capable of modulating striatal activity and triggering cGMP pathways linked to synaptic plasticity. The close proximity of the different neurones allows for multiple interactions of dopamine-glutamate for regulation of NO production.

Striatal nNOS interneurones receive asymmetric synapses from glutamatergic afferents and express NMDA in addition to AMPA and metabotropic glutamate receptors (Gracy & Pickel, 1997). NMDA receptors are a key element in facilitating synaptic plasticity changes in the central nervous system, and these glutamate-gated oligomeric ion channels are formed by co-assembly of members of three receptor subunit families; NMDA receptor I (NR1), NR2A-NR2D and NR3A-NR3B (Dingledine *et al.*, 1999; Nishi *et al.*, 2001). Activation of NMDA receptors causes an influx of calcium ions (Ca^{2+}) through the receptor channel which is an absolute requirement for LTP/LTD induction (Collingridge *et al.*, 2004). In turn this Ca^{2+} influx can stimulate the nNOS mediated synthesis of NO at the post-synaptic density provided the calcium ion concentration meets the 400nM required for calmodulin to bind to nNOS and hence activate the enzyme (Garthwaite *et al.*, 1989; Knowles *et al.*, 1989). Both nNOS and the NMDA receptor are bought into close proximity as they bind to the postsynaptic density protein PSD-95 so directly exposing nNOS to the Ca^{2+} (Tomita *et al.*, 2001). Indeed the intrastriatal infusion of NMDA has

been shown to activate NO efflux *in vivo* (Babu *et al.*, 1998; Crespi & Rossetti, 2004). NMDA receptor antagonists also appear much more effective when injected intrastriatally than when given systemically or injected into other structures of the basal ganglia in a rodent model of PD (Marin *et al.*, 1996). Subsequent production of NO can then influence cGMP levels via the activation of soluble guanylate cyclase (sGC) present in these cells (Boulton *et al.*, 1995). sGC acts as an NO receptor and its expression and activity are higher in the striatum than any other brain region (Hofmann *et al.*, 1977; Matsuoka *et al.*, 1992).

In the striatum, stimulation of the NO/cGMP pathway within corticostriatal fibres is capable of modulating processes such as LTD that has been linked to dyskinesia induction (Calabresi et al., 1999). It has recently been demonstrated that dopamine modulates striatal nNOS activity which becomes upregulated by activation of D1 receptors and down-regulated by D2 receptors dependent on ongoing NMDA receptor activity (Hoque *et al.*, 2010). Dopamine- glutamate interactions involved in regulation of nNOS activity within the striatum are likely to be complex as the neurones converge both at the level of NOS interneurones in addition to the principle medium-sized spiny neurons (Morello *et al.*, 1997; Sancesario *et al.*, 2000; Hidaka & Totterdell, 2001). Physiological dopaminergic modulation of NO signalling appears critical for execution of normal motor behaviour and inhibitors of nNOS have caused adverse motor effects in rodents including the induction of catalepsy and interference with fine motor control (Del Bel *et al.*, 2005).

1.4.3 NOS Inhibitors

The overproduction of NO is associated with a number of diseases hence the NOS pathway is a desirable target for the development of therapeutics. Consideration of NOS isoform selectivity is important to prevent undesirable side-effects and it is generally accepted that NOS inhibitors of medical value should avoid inhibition of eNOS owing to its critical role in maintaining vascular tone (Babu & Griffith, 1998a). For the purpose of this thesis inhibition of nNOS shall be focused upon from herein.

Early NOS inhibitors were guanidino amino acids being analogues of L-arginine showing little isoform selectivity. Several of these analogues, such as N-propargyl-L-arginine and L-thiocitrulline, acted as classic competitive inhibitors at the NOS active site with a rapid onset, demonstrating no progress with time or covalent modifications of the enzyme and full reversibility by dilution or dialysis (Frey *et al.*, 1994; Fast *et al.*, 1997). Mechanism-based inactivators were also developed similarly targeting the arginine binding site. N-methyl-L-arginine (L-NMA), was one such compound, binding with a tenfold higher affinity than L-arginine to the substrate site of NOS and slowly metabolised by active iNOS and nNOS resulting in irreversible inactivation of the enzyme (Feldman *et al.*, 1993; Olken & Marletta, 1993). N-nitro-L-arginine (L-NNA) is the active NOS inhibitor formed by hydrolysis from the inactive prodrug N-nitro-L-arginine methyl ester (L-NAME) and shows some selectivity towards nNOS and eNOS (Mayer & Andrew, 1998). Dissociation of the L-NNA-NOS complex is slow, so the drug is a highly potent tight-binding inhibitor of both constitutive NOS isoforms. Substitution of the guanidine group of L-arginine

analogues with a short hydrocarbon chain conferred greater selectivity producing *N*-(1-imino-3-butenyl)-L-ornithine (vinyl-L-NIO) which is a potent, mechanism based inhibitor that interacts with the heme cofactor of NOS and shows appreciable selectivity for nNOS (IC_{50} for nNOS = 0.1 μM, eNOS = 12 μM and iNOS = 60 μM) (Babu & Griffith, 1998b). However this compound did not appear to penetrate the blood-brain barrier (BBB).

Various heterocyclic modified L-arginine analogues have been shown to inhibit NOS including the nitroindazole group of fused heterocycles competitively interacting with the substrate binding site. 7-nitroindazole (7-NI) carries a net electronegative charge and consequently binds reversibly to the heme prosthetic group of NOS, subsequently interfering with tetrahydrobiopterin binding to the enzyme and preventing NO formation (Moore & Handy, 1997; Mayer & Andrew, 1998). Although *in vitro* data show 7-NI induces non-selective NOS inhibition (IC_{50} for nNOS = 8.3 μM, eNOS = 0.7 μM and iNOS = 57 μM), administration of 7-NI *in vivo* results in central nNOS inhibition without cardiovascular effects attributable to eNOS inhibition (Babbedge *et al.*, 1993). Its *in vivo* selectivity for nNOS is suggested to result from the xanthine-oxidase-mediated catabolism of 7-NI by intact endothelial cells preventing interaction with eNOS (Handy & Moore, 1998). However there is some evidence advocating that 7-NI does affect eNOS activity *in vivo* (Zagvazdin, 1996). Nevertheless 7-NI has been used as an effective central NOS blocker in a wide range of experiments, including studies of renal function, learning and memory, nociception and neuroprotection against MPTP-induced toxicity (Moore *et al.*, 1993a; Przedborski *et al.*, 1996; Li *et al.*, 2002; Wangensteen *et al.*, 2006).

Inhibitors lacking significant structural similarity to L-arginine but competitively binding to the same substrate site have also been developed, although they have often demonstrated poor cellular penetration (Paige & Jaffrey, 2007). The isothiourea derived ARR17477 (also referred to as ARL17477) based on a simple guanidine and amidine template showed potent and isoform selective nNOS inhibition both *in vitro* and *in vivo* (IC_{50} for nNOS = 0.035-0.07 μM, eNOS =1.6-3.5 μM and iNOS =0.33-5.0 μM), rapidly penetrating the brain and resulting in long-lasting inhibition (Reif *et al.*, 2000; Vallance & Leiper, 2002). ARR17477 has also proved effective in animal models of ischaemia (Zhang *et al.*, 1996; O'Neill *et al.*, 2000).

Northwestern University and Neuraxon have both endeavoured to create novel nNOS- specific inhibitors with increased selectivity in recent years but progress has been hindered by sub-optimal penetration of the BBB on moving into *in vivo* models, a property which would be essential for addressing diseases affecting the CNS (Joubert & Malan, 2011). Northwestern University patented a series of aminopyridines showing potent nNOS inhibition (IC_{50} for nNOS = 28 - 103 nM) and selectivity over eNOS (96-156-fold) and iNOS (19-52-fold) reported to possess improved oral bioavailability (Xue *et al.*, 2010b; Xue *et al.*, 2011).

1.4.4 NOS in PD and dyskinesia

The inhibition of nNOS has shown promise for neuroprotection in Parkinson's disease by counteracting the neurotoxic effects of excess NO at dopaminergic neurons (Hantraye *et al.*, 1996; Castagnoli *et al.*, 1997; Singh & Dikshit, 2007). However, on starting this thesis, despite strong implications for the involvement of NO in PD, nNOS inhibitors had not been investigated towards therapeutic benefit for dyskinesia.

In vivo post-mortem brain studies reveal a significant increase in nNOS mRNA expression in the dorsal two thirds of the subthalamic nucleus and the medial medullary lamina of the globus pallidus in patients with PD as compared to controls (Eve et al., 1998). Meanwhile there is a significant decrease in NOS expression in the putamen in Parkinson's disease (Eve et al., 1998). These divergences support a role for changes in NO synthesis within the basal ganglia in PD. Additionally increased levels of nitrite and the NO second messenger cGMP have been found in the serum of PD patients (Qureshi *et al.*, 1995; Chalimoniuk & Stepien, 2004). nNOS related susceptibility genes have also been identified in PD patients (Hancock *et al.*, 2008). Although the direction of change within these investigations appears inconsistent, the data collectively suggest non-physiological levels of NO and related molecules to be present in PD.

Further evidence from PD animal models substantiates these findings and striatal nNOS activity is seen to be significantly depressed following nigrostriatal deafferentation in the 6-OHDA lesioned rat (De *et al.*, 2000; Sancesario *et al.*, 2004). Indeed corticostriatal plasticity is lost after 6-OHDA-lesioning in rats and chronic L-dopa treatment can restore physiological synaptic transmission, but only for animals which do not develop AIMs suggesting enduring aberrant storage processes exist in dyskinetic animals (Picconi *et al.*, 2003). A further study indicates that the inability to restore regular synaptic plasticity by L-dopa treatment is specific to medium spiny neurons of the direct striatonigral pathway (Belujon *et al.*, 2010). The same study also showed that in dyskinetic rats the regulation of synaptic plasticity in direct and indirect pathways is out of balance. Additionally L-dopa has been shown to cause a significant rise in the production of NO, as measured by NO_3^- levels, within the striatum (Itokawa *et al.*, 2006). Furthermore, in our laboratories, chronic L-dopa treatment of 6-OHDA-lesioned rats resulted in increased expression of striatal nNOS mRNA on the lesioned side of the brain compared to drug naïve control 6-OHDA lesioned animals (Iravani et al., unpublished data). Indeed, glutamatergic overactivity at NMDA receptors may prompt these long-term synaptic changes within the striatum which inappropriately modulate motor function.

Interaction between dopamine D1 and D2 receptors and NMDA receptors is involved in modulation of nNOS activity and the control of striatal plasticity (Hoque *et al.*, 2010). These may occur via calcium–calmodulin-dependent protein kinase II (CaMKII). This protein has been seen to function as a signal integrator downstream of dopamine and glutamate receptors at the post-synaptic density of striatal spiny neurons and appears to be hyperphosphorylated and accompanied by a higher recruitment of activated α-CaMKII to the regulatory NR2 NMDA receptor subunits in PD models (Picconi et al., 2004).

Abnormal NO and nNOS activity may therefore be characteristic of PD and dopaminergic treatment may subsequently 'normalise' levels except where dyskinesia develops the balance is upset once again and leads to further downstream molecular changes.

In light of evidence for glutamatergic overactivity within the basal ganglia in dyskinesia coupled with the ability of glutamate to activate NMDA receptors, causing an influx of calcium ions and stimulation of nNOS mediated synthesis of NO at post-synaptic densities within the striatum linked to permanent changes in synaptic plasticity (see also section 1.4.2), a novel opportunity may be provided for the intervention of mechanisms underlying dyskinesia induction and expression.

1.5 Thesis hypothesis

The identification of adjunctive agents which can effectively control the development of dyskinesia and the expression of established dyskinesia in PD without compromising the beneficial behavioural effects afforded by dopaminergic medication would be valuable in the clinic. The biochemical changes underlying dyskinesia are yet to be fully elucidated but there is strong evidence for the involvement of NO produced by nNOS suggesting that its reduction may alleviate or even prevent motor complications. Thus it is hypothesised that NO produced by nNOS contributes to the occurrence and evolution of dyskinesia in PD, and this can be controlled by nNOS inhibition.

1.6 Thesis aims

To test this hypothesis, the studies described in this thesis investigated the role of nNOS inhibitors as potential antidyskinetic agents in established *in vivo* models with the capacity to demonstrate abnormal involuntary movements akin to dyskinesia.

More specifically the thesis aimed:

1. To establish doses of nNOS inhibitor required to cause a significant reduction in central nNOS activity

2. To investigate the role of nNOS in the expression of pre-existing dyskinesia in 6-OHDA-lesioned rats and MPTP-treated primates

3. To investigate the role of nNOS in the induction of dyskinesia in 6-OHDA-lesioned rats and MPTP-treated primates

Chapter 2 : Materials and methods

2.1 Introduction

The aim of the studies reported in this thesis was to investigate the potential for nNOS inhibitors to reduce dyskinesia using *in vivo* models of PD. For this purpose two well established animal models were employed; the 6-OHDA-lesioned rat and the MPTP-treated marmoset. Both of these models provide a functional basis for assessing motor changes associated with degeneration of dopaminergic pathways as seen in the human condition of PD. These models were specifically used to probe the processes of expression of dyskinesia in primed animals, and the development of dyskinesia in drug naïve animals, as well as in combination with nNOS inhibitors. The general methodologies utilised for these investigations are described below.

2.2 The 6-OHDA rat model:

2.2.1 Introduction

The neurotoxin 6-OHDA has become one of the most commonly used toxins for modelling PD in rodents since Ungerstedt first established a working model in 1968. It principally exerts effects on catecholaminergic neurones and stereotaxic injection of 6-OHDA into the medial forebrain bundle (MFB) causes loss of dopaminergic neurones projecting from the SNpc to the striatum of up to 99 % on the lesioned side. The unilaterally 6-OHDA-lesioned rat shows minimal overt symptoms following recovery from surgery, however when challenged with agents affecting the dopaminergic system, such as amphetamine or apomorphine, a circling response is elicited. The direction and severity of the response is dependent upon the pharmacological mechanism of the drug and also the extent of the lesion (Schwarting & Huston, 1996; Deumens *et al.*, 2002). Furthermore, following chronic administration of dopaminergic agents abnormal involuntary movements (AIM's) tend to develop, provided the loss of nigrostriatal dopamine neurones exceeds 80 % (Cenci, 2007). These include axial, limb and oral movements all of which predominantly manifest on the body side contralateral to the lesion. As in the MPTP primate model (see later), 6-OHDA-lesioned rats respond to drugs shown to suppress dyskinesia in man, hence providing strong validation for the model (Lundblad *et al.*, 2002). Thus the behavioural phenomena characteristic to drug exposure in 6-OHDA-lesioned rats afford an accessible testing bed for potential therapeutic interventions for PD.

2.2.2 Animal husbandary

Male Wistar rats (225-250 g; Harlan, UK or B & K, UK) were used in all procedures and housed 2-3 per cage in the Biological Service Unit, King's College London. Room temperatures were maintained at 19-21 ˚C at 55 % humidity with a 12 h light-dark cycle and animals had free access to pelleted food and water. Animals had a minimum five day acclimatisation period from arrival prior to any procedure. Experiments were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986 under Home Office project licence no. 70/6019 or 70/6898.

2.2.3 6-OHDA lesion induction

A unilateral injection of 6-OHDA hydrochloride was administered into the rat brain using standard stereotaxic techniques. Animals were initially anaesthetised by placing in an induction chamber with isofluorane 4-5 % in 95 % O_2 and 5 % CO_2 until observable loss of the 'righting reflex'. They were then transferred into a Kopf stereotaxic frame and maintained under 2-3 % isofluorane in 95 % O_2 and 5 % CO_2, with ear bars inserted symmetrically and the incisor bar set to -3.3 mm in accordance with Paxinos & Watson (1986). A thermostatic heated blanket was placed under the animal to maintain a temperature of 37 ˚C throughout surgery. The rat's head was shaved, sterilised with an alcohol swab, EMLA cream applied and a central anterior-posterior incision made with a disposable scalpel. The skull was exposed by retracting the skin and tissue, and a hole made into the skull with a hand-drill above the left MFB according to the coordinates of Paxinos and Watson (1986); A-P:-2.6 mm, M-L: +2.0 mm, as measured from bregma (see **Figure 2-1**). The 26S gauge needle of a Hamilton syringe was gently lowered to V:-8.8 mm and 6-OHDA hydrochloride (8 µg free base in 4 µl 0.9 % saline containing 0.05 % ascorbic acid) injected at a rate of 1 µl per minute using an automated micro-injection pump (Harvard Apparatus). The needle remained in place for a further 4 min before withdrawal over another 4 min period. Animals then received a 5 ml intraperitoneal (i.p.) injection of 5 % glucose in 0.9 % saline, the wound was cleaned with swabs and the skin sutured using three to four separate stitches of dissolvable vicryl suture.

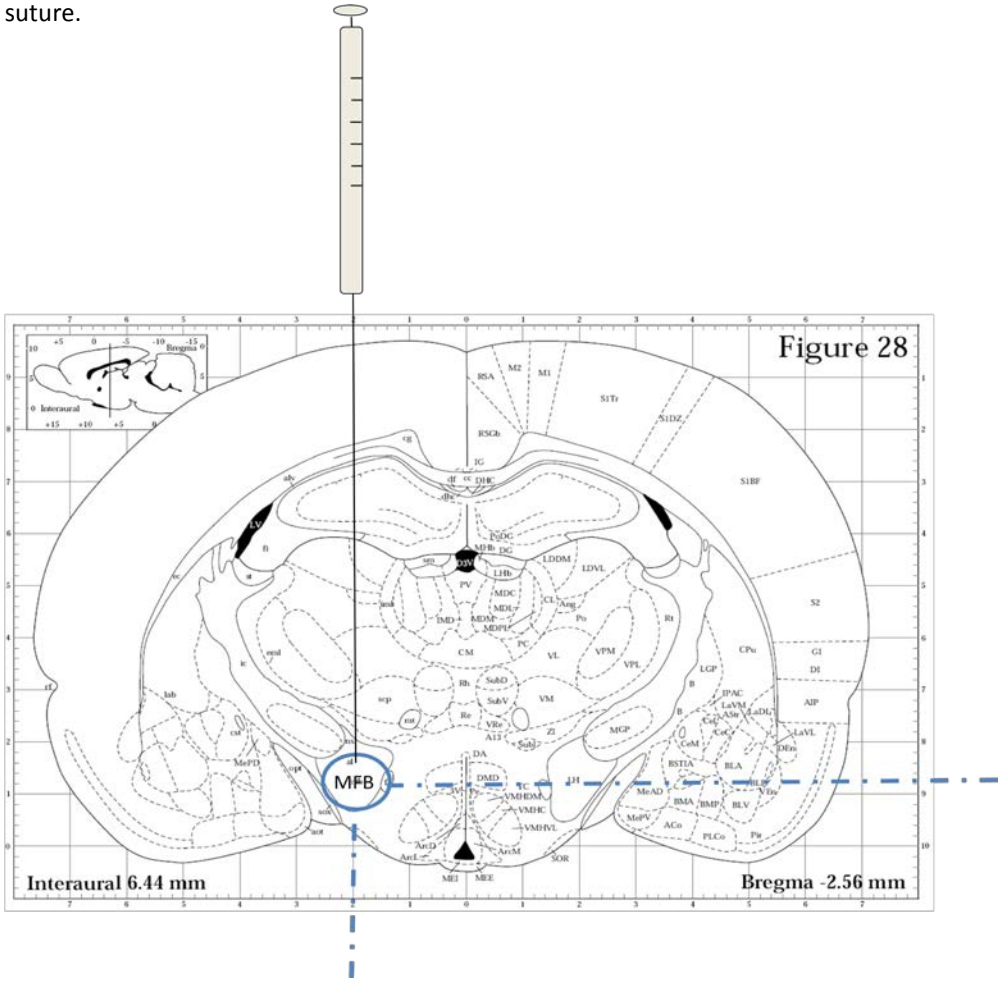

Figure 2-1 Co-ordinates used for microinjection of 6-OHDA into the MFB; as taken from The Rat Brain: In Stereotaxic Coordinates (Paxinos & Watson, 1986).

2.2.4 Post-operative care

Rats were placed in a warmed incubation chamber to recover from anaesthesia before being returned to home cages. Post-surgery, softened rat food mixed with warm water was provided to encourage eating, and body weights were monitored until they returned to pre-operative levels or stabilised.

2.3 The 6-OHDA rat model: Behavioural assessment

2.3.1 Automated measurement of rotational activity

The extent of 6-OHDA lesion was assessed at three-four weeks post-surgery. Following a 30 minute habituation period to rotometry arenas, animals were administered amphetamine sulphate (2.5 mg/kg i.p. in 0.9 % saline). Tracksys Video monitoring Software and Equipment was used to measure rotational movement of the rat by detection of the tip of the head, centre point and start of the tail (see **Figure 2-2**) for a 3 h period. Ethovision software processed the readings to provide data for rotations per minute for individual animals. Rats exhibiting 7 ipsilateral turns per minute or more at peak activity, corresponding to a lesion of >50 % (Hefti *et al.*, 1980), were taken forward for further experiments (see **Figure 2-3**).

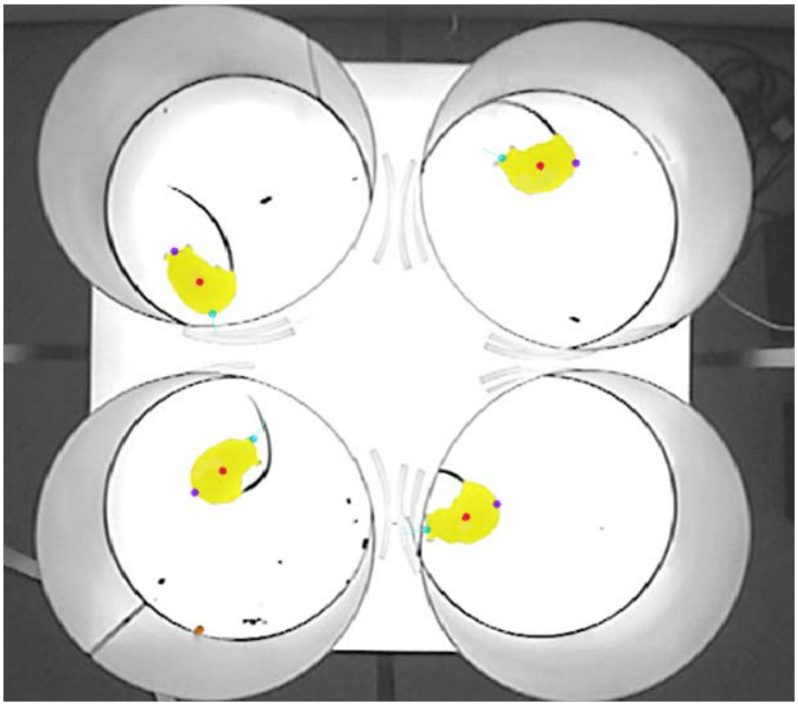

Figure 2-2 Tracksys video monitoring of rotational rat behaviour; animals (n=4) are automatically detected in arenas and highlighted (yellow) with tip of the head (turquoise), centre point (red) and tail join to body (purple) by the software.

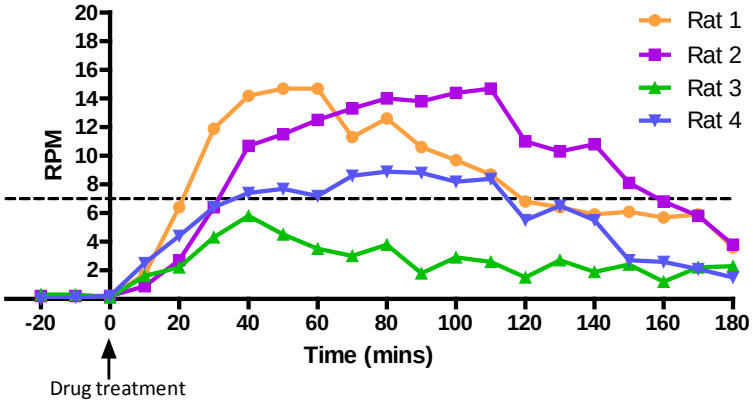

Figure 2-3 Rotations per minute (RPM) typical data as measured by Tracksys rotometry software and equipment. Animals were treated with amphetamine sulphate 2.5 mg/kg i.p. and net ipsilateral RPM's meaned per 10 min for each rat. Data shown as individual RPM's for each rat (n=4). Interquartile range was typically 5RPM. The dotted line indicates the threshold value for peak RPM's used to select animals with a full MFB lesion. In this case rats 1, 2 & 4 were taken forward for AIM's experiments.

2.3.2 Dyskinesia assessment

2.3.2.1 Establishing dyskinesia

Following amphetamine washout a group of 6-OHDA-lesioned rats, showing ≥7 RPMs upon rotational assessment, were dosed daily with L-dopa methyl ester (6.25 mg/kg + benserazide 15 mg/kg) for a period of 22 days to establish stable dyskinesia levels as measured by AIMs (for explanation of AIMs refer to 2.3.2.2). Animals were scored for dyskinesia at baseline (day 0) and, from day 1 of dosing, on every third day as shown in **Figure 2-4**. Beyond the priming period animals were dosed twice weekly with L-dopa (6.25 mg/kg + benserazide 15 mg/kg) to ensure a persistent dyskinetic response until subsequent studies commenced.

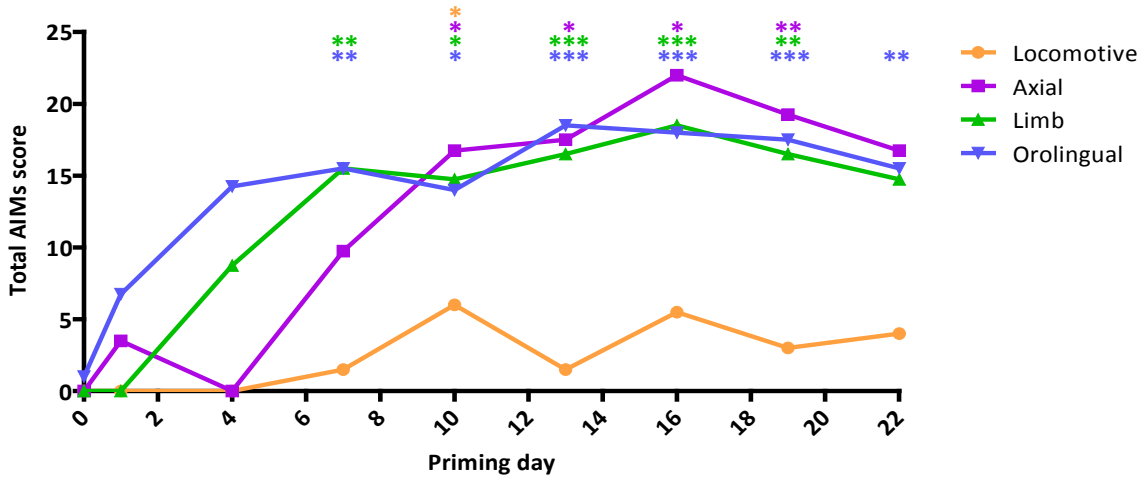

Figure 2-4 AIMs development during L-dopa priming of 6-OHDA-lesioned rats post rotational screening; animals (n=10) were treated with L-dopa methyl ester (6.25 mg/kg + benserazide 15 mg/kg) on a daily basis for 22 consecutive days and scored for locomotive, axial, limb and orolingual AIMs every third day; total AIMs score data (for a 3 h scoring period) are presented as medians for each behavioural test day and interquartile ranges of these parameters were typically 10 AIMs units; ***p<0.001, **p<0.01, *p<0.05 compared to Day 0 baseline score for each AIMs subtype (colours used refer to key); Friedman's test followed by post hoc Dunn's test.(Data taken from Chapter 4).

2.3.2.2 AIMs scoring

All AIMs experiments were carried out between the hours of 8.30 am and 4.00 pm. Rats were placed individually into transparent rectangular cages, measuring 20 cm x 36 cm x 18 cm (See **Figure 2-5**), one hour before drug administration to acclimatise. Baseline scores were taken 20 min and 5 min prior to dosing. Following drug treatment AIMs were then scored over a duration of 5 min every 15 min for up to 210 min. Scoring was carried out by a trained observer blinded to treatment. Blinding was achieved by coding drug solutions prior to dosing, and administering these according to a modified latin square design such that every animal received every treatment over the course of the study (unless otherwise stated).

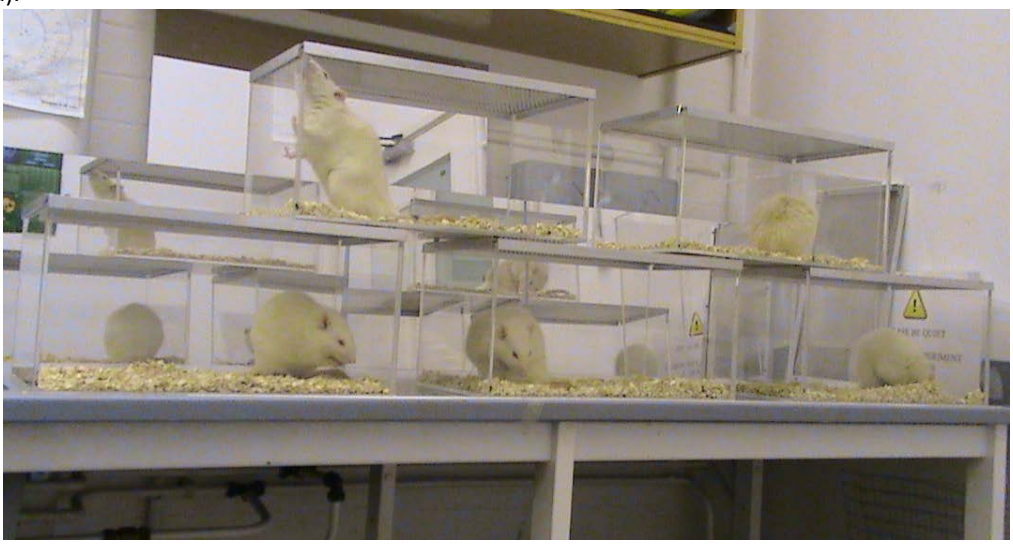

Figure 2-5 Transparent cages set up for measuring AIMs in 6-OHDA lesioned rats; mirrors are placed behind cages to increase visibility of rat behaviour as animals move about.

To assess dyskinesia in rats four different subtypes of AIMs were scored according to anatomical origin of the involuntary movement, and each rated on a scale of 0-4 (See **Table 2-1** and **Table 2-2**). The scoring system was based on that devised by Cenci and colleagues (Cenci *et al.*, 1998; Winkler *et al.*, 2002; Dekundy *et al.*, 2007) with minor modifications. Locomotive, axial, limb and orolingual AIMs (as exemplified in **Figure 2-6**) were assessed by observing the rats' behaviours in transparent cages positioned as in **Figure 2-5**, in real-time. As a general rule each form of dyskinesia was rated as absent (score 0), mild (score 1), moderate (score 2), marked (score 3) or severe (score 4), as described in **Table 2-2**. In order to increase the sensitivity of the rating scale incremental scores of 0.5 were also employed between 0-4 where applicable. Individual scores for axial, limb and orolingual AIMs sub-types were summed together for each assessment period to give an overall dyskinesia level, denoted as 'ALO AIMs' (axial + limb + orolingual score). Exhibition of these three sub-types in particular are considered to best mimic dyskinesia according to published literature (Dekundy *et al.*, 2007). ALO AIMs were regarded as absent (score = 0), mild (1-3), moderate (4-6), marked (7-9) or severe (10-12). Meanwhile locomotive scores were considered separately as is commonplace in the literature due to controversy as to whether locomotor behaviour truly represents dyskinesia (Lundblad *et al.*, 2002). The duration of ALO AIMs and AIM's subtypes calculated for locomotive, axial and limb AIMs took into account all score periods gaining above 0 and in the case of orolingual AIMs included those gaining above 1 (due to animals

frequently showing mild orolingual AIMs during baseline assessments i.e. ~0-1). A mean of the two baseline scores was taken on each assessment day to give a single baseline score at t=0.

Table 2-1 AIMs classification

AIM's subtype	Description
Locomotive	Rotations contralateral to lesion
Axial	Dystonic or choreic torsion with lateral deviation of the head, neck and/or trunk towards the side contralateral to the lesion
Limb	Jerky and/or dystonic movements of the forelimb contralateral to the lesion
Orolingual	Empty jaw movements, facial muscle twitching and/or tongue protrusion, more pronounced on side contralateral to lesion

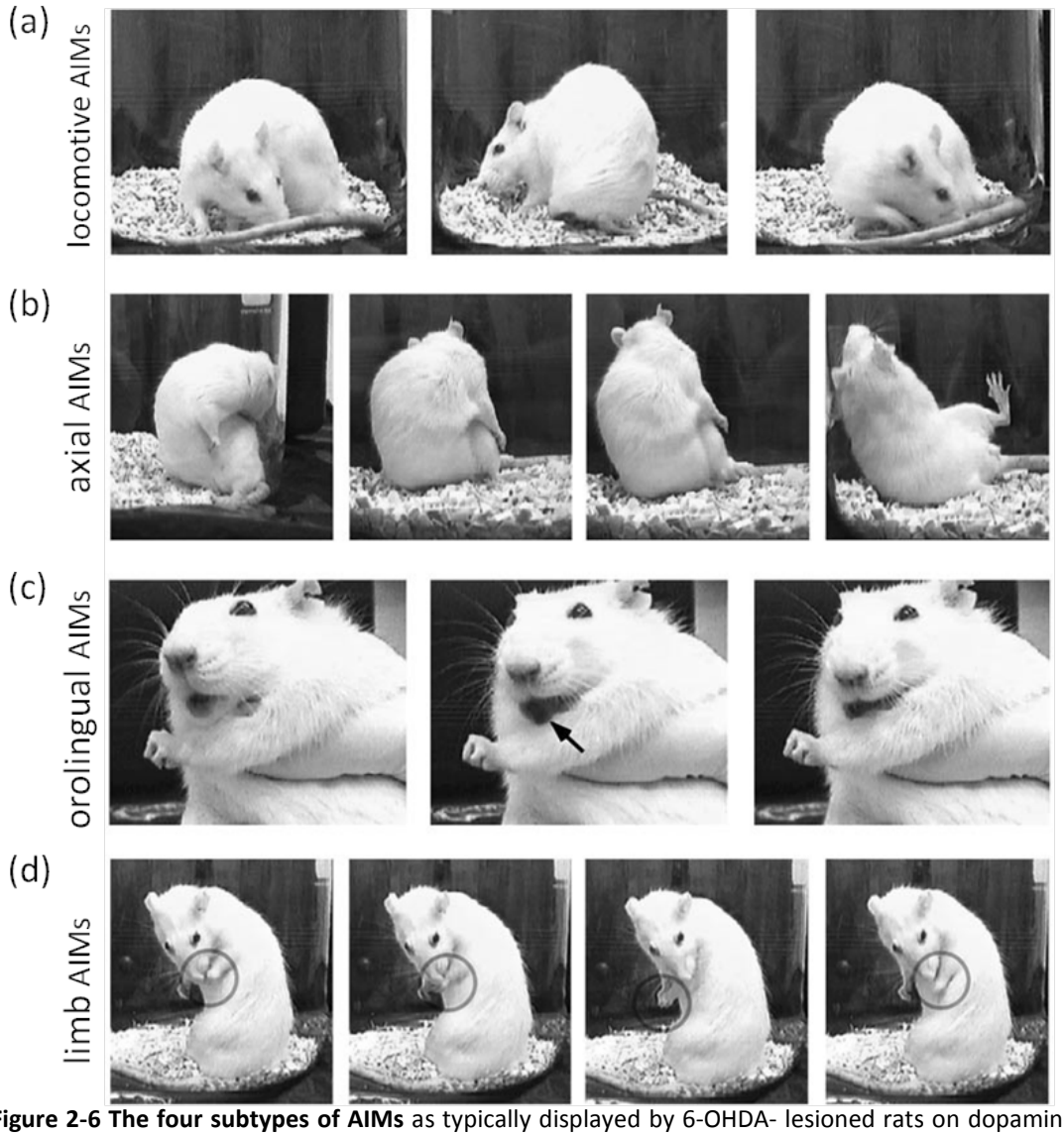

Figure 2-6 The four subtypes of AIMs as typically displayed by 6-OHDA- lesioned rats on dopaminergic drug treatment; locomotive (a), axial (b), orolingual (c) and limb (d), (Winkler et al., 2002).

Table 2-2 AIMs scoring scale

AIM's subtype	AIM's score	Description
Locomotive		
	0	Absent
	1	Occasional (<50 % of observation time)
	2	Frequent (>50 % of observation time)
	3	Continuous but interruptible by external stimuli
	4	Continuous and not interruptible by external stimuli
Axial		
	0	Absent
	0.5	Brief periods of lateral deviation of head and neck at ~30 ˚ angle
	1	Consistent lateral deviation of head and neck at ~30 ˚ angle
	1.5	Consistent lateral deviation of head and neck at ~30 ˚ angle with brief periods of deviation of head and neck at 30 ˚<angle≤60 ˚ angle
	2	Contralateral deviation of head and neck at 30 ˚<angle≤60 ˚ angle
	2.5	Contralateral deviation of head and neck at 30 ˚<angle≤60 ˚ angle with brief periods of deviation of head, neck and upper trunk at 60 ˚<angle≤90 ˚ angle
	3	Contralateral deviation of head, neck and upper trunk at 60 ˚<angle≤90 ˚ angle
	3.5	Contralateral deviation of head, neck and upper trunk at >90 ˚ angle with some loss of balance
	4	Torsion of head, neck and trunk at >90 ˚ angle with persistent loss of balance
Limb		
	0	Absent
	0.5	Brief and fleeting small paw movements around a fixed position
	1	Small paw movements around a fixed position
	1.5	Small paw movements with occasional low amplitude translocation of limb
	2	Low amplitude paw movements with translocation of distal limb
	2.5	Low amplitude paw movements with translocation of proximal and distal limb
	3	Translocation of whole limb with contraction of shoulder muscles
	3.5	Translocation of whole limb with some contraction of shoulder muscles at high frequency or maximal amplitude
	4	Vigorous limb and shoulder movement of high frequency and maximal amplitude
Orolingual		
	0	Absent
	0.5	Occasional (<25 % of observation time)
	1	Occasional (<50 % of observation time)
	1.5	Occasional (present 50 % of observation time)
	2	Frequent (>50 % of observation time)
	2.5	Frequent (>75 % of observation time)
	3	Continuous but interruptible by external stimuli
	3.5	Continuous but sometimes interruptible by external stimuli
	4	Continuous and not interruptible by external stimuli

2.3.2.3 Characterisation of the AIM's model

Introduction. In combination with L-dopa, amantadine (a weak NMDA antagonist), 8-Hydroxy-2-(dipropylamino)tetralin (8-OHDPAT; a 5-HT1A agonist), and MK-801 (a non-competitive NMDA antagonist) have all been shown to reduce AIMs according to published literature (Dekundy *et al.*, 2007; Dupre *et al.*, 2008). For characterisation of the AIM's model, as established by Cenci *et al.* (1998), these three compounds were tested with L-dopa in cross-over studies in dyskinesia primed 6-OHDA-lesioned rats (n=7-8).

Method. Primed 6-OHDA-lesioned rats (as described in 2.3.2.1) were scored for AIMs in transparent cages (as described in section 2.3.2.2). After baseline scores were taken, they were then treated with both L-dopa methyl-ester (6.25 mg/kg + benserazide 15 mg/kg i.p.) and also either saline (0.9 % i.p.), amantadine hydrochloride (20 or 40 mg/kg i.p.), MK-801 (0.4 mg/kg i.p.), or 8-OHDPAT (0.6 mg/kg i.p.). Doses of amantadine, MK-801 and 8-OHDPAT utilised were chosen based on referenced literature in combination with previous findings from our lab. All compounds were dissolved in saline (0.9 %). Graphs showing time courses and totals were plotted as medians (n=7-8). Total AIMs scores were calculated from the time courses by AUC (Graphpad Prism version 5.0) using the trapezoid method where each successive 15 min was labelled as a single time-bin. Data were analysed by Friedman's test followed by post hoc Dunn's test. See **Figure 2-7**, **Figure 2-8**, **Figure 2-9** & **Figure 2-10**.

Results. L-dopa (6.25 mg/kg + benserazide 15 mg/kg) plus vehicle caused animals to exhibit axial AIMs from 15-180 min after dosing peaking at a score of 3.5 from 60-120 min. Limb and orolingual AIMs were seen from 15 min onwards for up to 180 min both peaking at a score of 2. ALO AIMs reflected these behaviours with summed AIMs scores increasing above baseline levels from 15 min up until 180 min, these peaked with an ALO AIMs score of 7 at 90 min. Meanwhile, locomotive AIMs lasted until 165 min but were less intense than the other three AIMs subcategories with a peak score of 1.5.

Amantadine 20 mg/kg had no significant effect on any of the four AIM's subtypes exhibited by L-dopa (6.25 mg/kg). However amantadine 40 mg/kg caused a significant reduction in axial and locomotive AIMs and a slight reduction in orolingual AIMs at 60-150 min after drug dosing. Amantadine caused a stable and long lasting reduction (from 15-180 min), in L-dopa induced ALO AIMs with significant effects seen at the highest dose of 40 mg/kg.

MK-801 0.4 mg/kg reduced all four AIM's subtypes, compared to L-dopa plus vehicle effects, for the majority of the 3 h scoring period indeed inducing axial and locomotive AIMs on the ipsilateral body side (denoted by the negative scores). Considering total AIM's, MK-801 reduced ALO AIMs on the contralateral side at all time points following dosing.

8-OHDPAT 0.6 mg/kg markedly reduced all AIM's subtypes relative to L-dopa plus vehicle effects, causing axial and locomotive AIMs to be ipsilateral to the lesioned side within the first 60 min after drug treatment. Orolingual, ALO AIMS and locomotive AIMs were significantly reduced by 8-OHDPAT (0.6 mg/kg) compared to L-dopa plus vehicle treatment across the observation period.

Conclusion. These results are in agreement with the reductions in L-dopa induced dyskinesia demonstrated in the AIMs model with amantadine, MK-801 and 8-OHDPAT as cited in the literature. Strictly speaking locomotive AIMs are not considered to be specific to dyskinesia per se (e.g. Lundblad et al., 2002), therefore more focus will be placed on ALO AIMs for the purpose of this thesis for which effects of drug treatments on dyskinesia in particular are of principal interest.

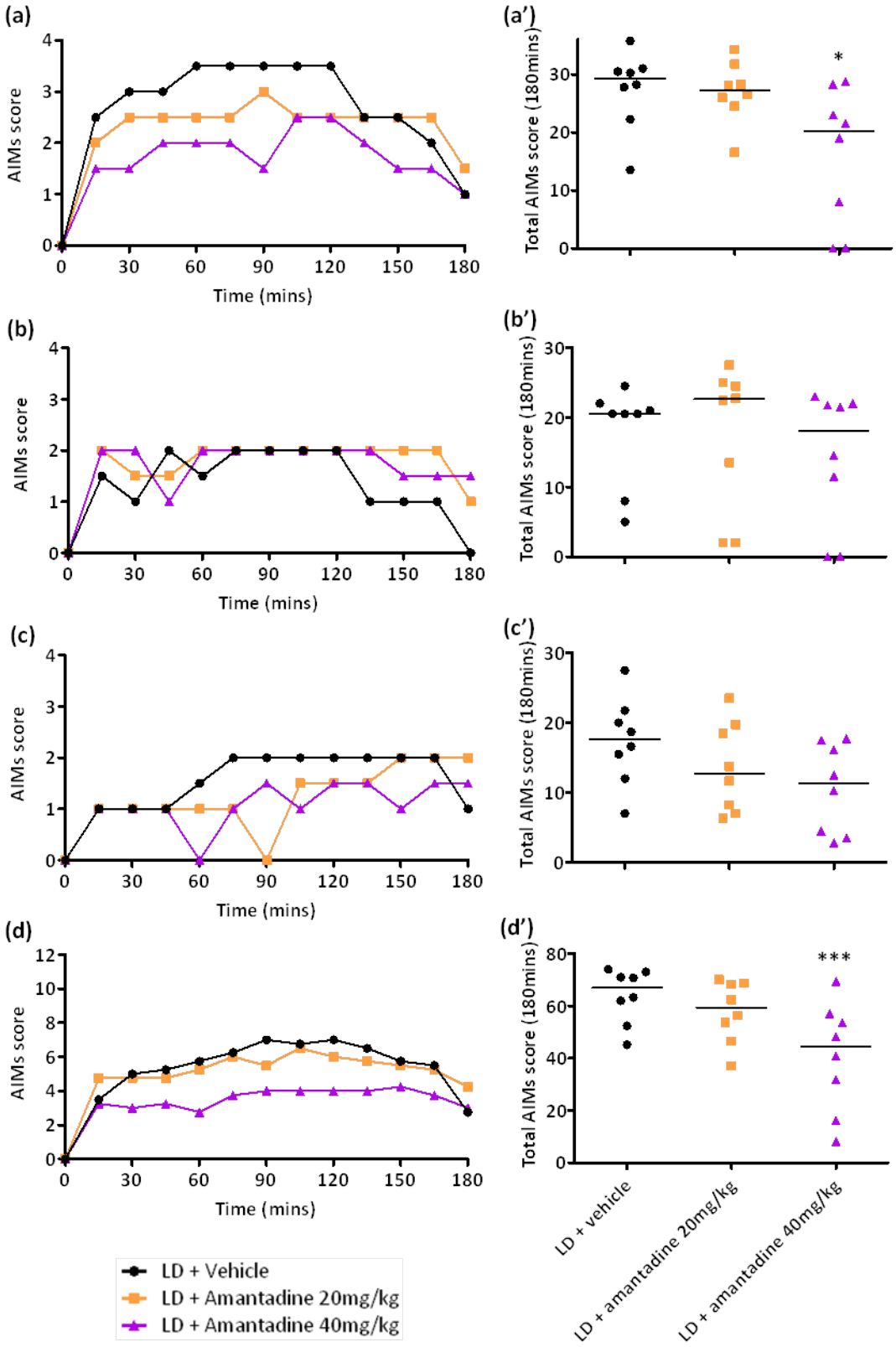

Figure 2-7 Amantadine AIMs characterisation axial, limb, orolingual and ALO data following L-dopa (LD; 6.25 mg/kg plus benserazide 15 mg/kg i.p.) plus vehicle or amantadine 20 and 40 mg/kg i.p. treatment. Data are presented as medians (n=8); Time-course of AIMs **(a)** axial, **(b)** limb, **(c)** orolingual and **(d)** ALO, and also individual values; Total AIMs **(a')** axial, **(b')** limb, **(c')** orolingual and **(d')** ALO; *p<0.05, ***p<0.001, compared to L-dopa plus vehicle treatment; Friedman's test followed by post hoc Dunn's test.

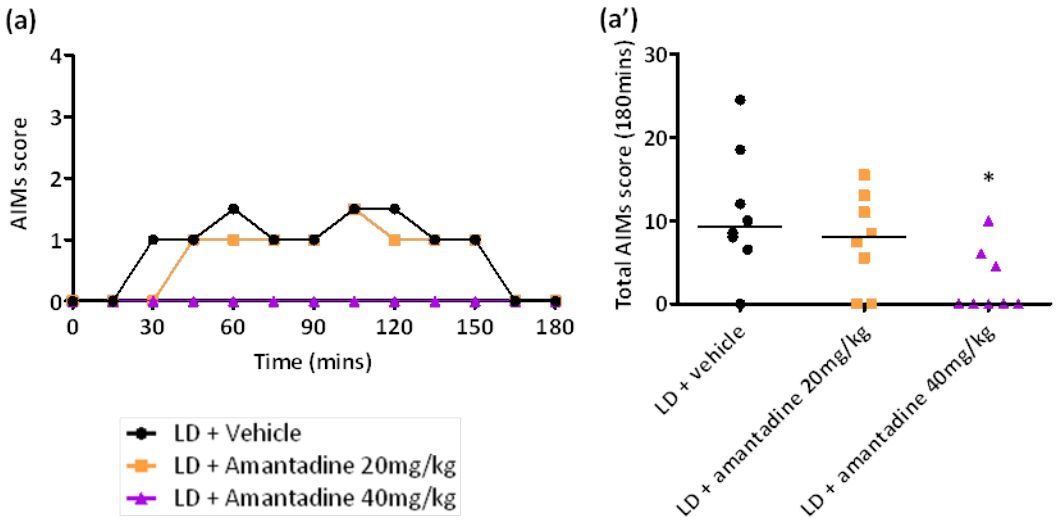

Figure 2-8 Amantadine AIMs characterisation locomotive data following L-dopa (LD; 6.25 mg/kg plus benserazide 15 mg/kg i.p.) plus vehicle or amantadine 20 and 40 mg/kg i.p. treatment. Data are presented as medians (n=8); **(a)** Time-course of AIMs, and also individual values; **(a')** Total AIMs; *p<0.05, compared to L-dopa plus vehicle treatment; Friedman's test followed by post hoc Dunn's test.

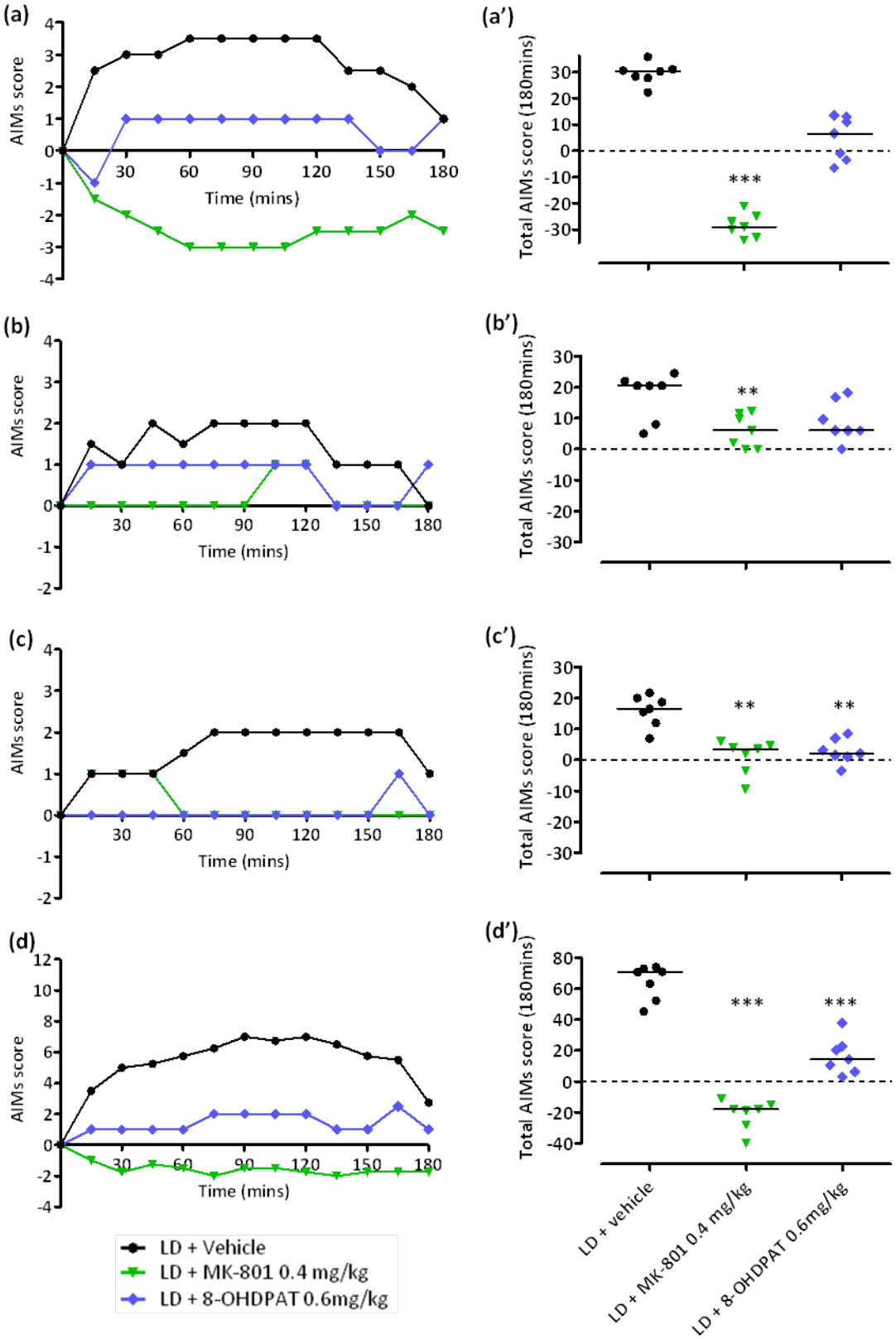

Figure 2-9 MK-801 and 8-OHDPAT AIMs characterisation axial, limb, orolingual and ALO data following L-dopa (LD; 6.25 mg/kg plus benserazide 15 mg/kg i.p.) plus vehicle, MK-801 0.4 mg/kg i.p. or 8-OHDPAT 0.6 mg/kg i.p. treatment. Data are presented as medians (n=7); Time-course of AIMs **(a)** axial, **(b)** limb, **(c)** orolingual and **(d)** ALO and also individual values; Total AIMs; **(a')** axial, **(b')** limb, **(c')** orolingual and **(d')** ALO; *p<0.05, **p<0.01, ***p<0.001, compared to L-dopa plus vehicle treatment; Friedman's test followed by post hoc Dunn's test.

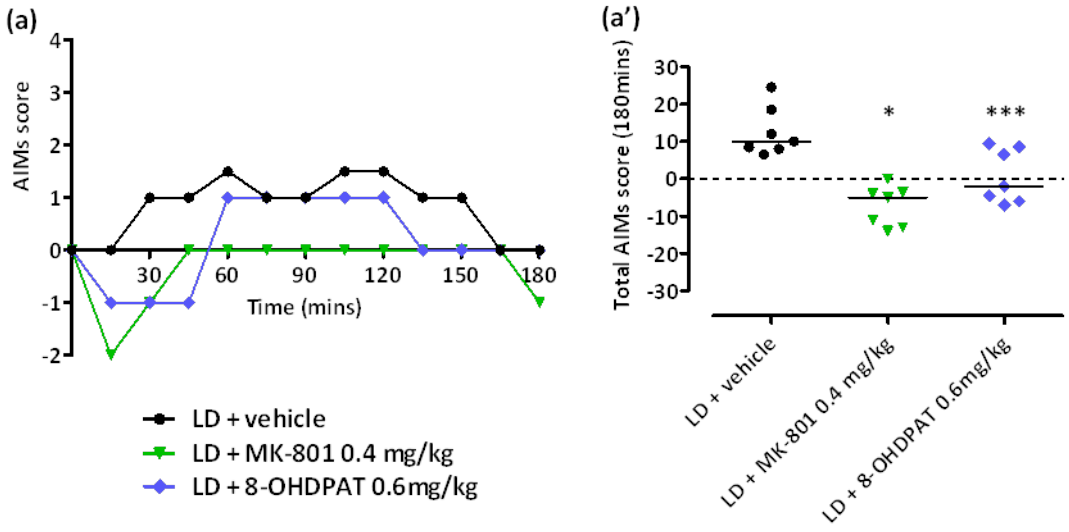

Figure 2-10 MK-801 and 8-OHDPAT AIMs characterisation locomotive data following L-dopa (LD; 6.25 mg/kg plus benserazide 15 mg/kg i.p.) plus vehicle, MK-801 0.4 mg/kg i.p. or 8-OHDPAT 0.6 mg/kg i.p. treatment. Data are presented as medians (n=7); **(a)** Time-course of AIMs, and also individual values; **(a')** Total AIMs; *p<0.05, ***p<0.001, compared to L-dopa plus vehicle treatment; Friedman's test followed by post hoc Dunn's test.

2.3.2.4 Establishing L-dopa and ropinirole doses for AIMs studies

Introduction Both L-dopa and dopamine agonists such as ropinirole can lead to dyskinesia expression after long-term use. To investigate the potential effects of nNOS inhibitors on resulting dyskinetic behaviour it was necessary to determine the optimal doses of dopaminergic agents to be used in AIMs investigations.

Method A group of primed 6-OHDA-lesioned rats (n=7-8) exhibiting stable AIMs (as described in 2.3.2.1), was administered a range of doses of L-dopa or ropinirole to provide a dose-response curve for both drugs. A cross-over study with a modified latin-square design was employed for each dopaminergic drug including a minimum 2 day drug washout between doses. Animals were treated with L-dopa methyl-ester (+ benserazide 15 mg/kg i.p.) (3.125, 6.25, 9.375, or 12.5 mg/kg i.p.), or saline (0.9 %) at 1ml/kg and scored for AIMs in transparent cages as described in section 2.3.2.2**.** Subsequently animals were treated with ropinirole (0.1, 0.2, 0.3, 0.4, or 0.5 mg/kg i.p.), or saline (0.9 %) at 1 ml/kg and AIMs were assessed as for the L-dopa treatments. As for characterisation of the AIM's model ALO AIM's time-course graphs, and totals, represented by area-under-the-curve (AUC), were plotted as medians (n=8). Data were analysed by Friedman's test followed by post hoc Dunn's test.

Results As seen in **Figure 2-11**, L-dopa (+ benserazide 15 mg/kg i.p.) at all doses (3.125, 6.25, 9.375 and 12.5 mg/kg) caused animals to exhibit dyskinesia as measured by ALO AIMs from 15 min onwards. ALO AIMS lasted up to 135 min for the lowest L-dopa dose (3.125 mg/kg) and up to 180 min for the highest doses (9.375 and 12.5 mg/kg). Scores peaked at 30-60 min after dosing reaching a maximum of 7-8 for all doses and remaining at these levels over a dose-dependent time frame increasing in duration with escalating dose. Locomotive AIMs were exhibited from 15-30 min onwards lasting for a time period which increased dose-dependently, and all doses resulted in a peak score of 1.5 except for the highest dose which peaked similarly at 2. In comparison, vehicle treatment induced minimal ALO AIMs at intermittent time periods lasting for no longer than two consecutive scoring sessions and peaking at a total score of 1, and locomotive AIMs were not seen throughout the assessment period. Only L-dopa doses of 6.25, 9.375 and 12.5 mg/kg resulted in significant behavioural manifestation of ALO and locomotive AIMs compared to vehicle treatment.

As seen in **Figure 2-12** all ropinirole doses (0.1, 0.2, 0.3, 0.4 and 0.5 mg/kg) induced an increase in ALO AIMs as compared to vehicle treatment manifesting at the 15 min score period and lasting up until 75 min after drug treatment for the lowest ropinirole dose (0.1 mg/kg) and until 145 min for the highest dose (0.5 mg/kg). ALO AIMs reached a peak after 15-30 min and duration of effect ranged from between 90-150 min. All doses from 0.2 mg/kg upwards resulted in peak ALO AIMs scoring 6-7, whilst at 0.1 mg/kg a peak score of 4 was exhibited. Meanwhile locomotive AIMs peaked at a score of 1-2 for all ropinirole doses and at 0.4 mg/kg lasted for a maximum period of 60 min. Compared to vehicle only treated animals, ropinirole doses from 0.2-0.5 mg/kg caused significant ALO AIMs and 0.4-0.5 mg/kg caused significant locomotive AIMs. Vehicle treatment did not appear to induce ALO or locomotive AIMs overall.

Conclusion Both L-dopa and ropinirole administration induce AIMs in 6-OHDA-lesioned primed rats over a time-course which lengthens with escalating dose. Peak AIMs scores show less dependence on dose. Compared to L-dopa, ropinirole is seen to be shorter acting with a smaller window of dyskinesia. L-dopa 6.25 mg/kg and ropinirole 0.2 mg/kg were selected for further investigations based on these data and in keeping with doses employed in the literature (Cenci *et al.*, 1998; Ravenscroft *et al.*, 2004; Marin *et al.*, 2009).

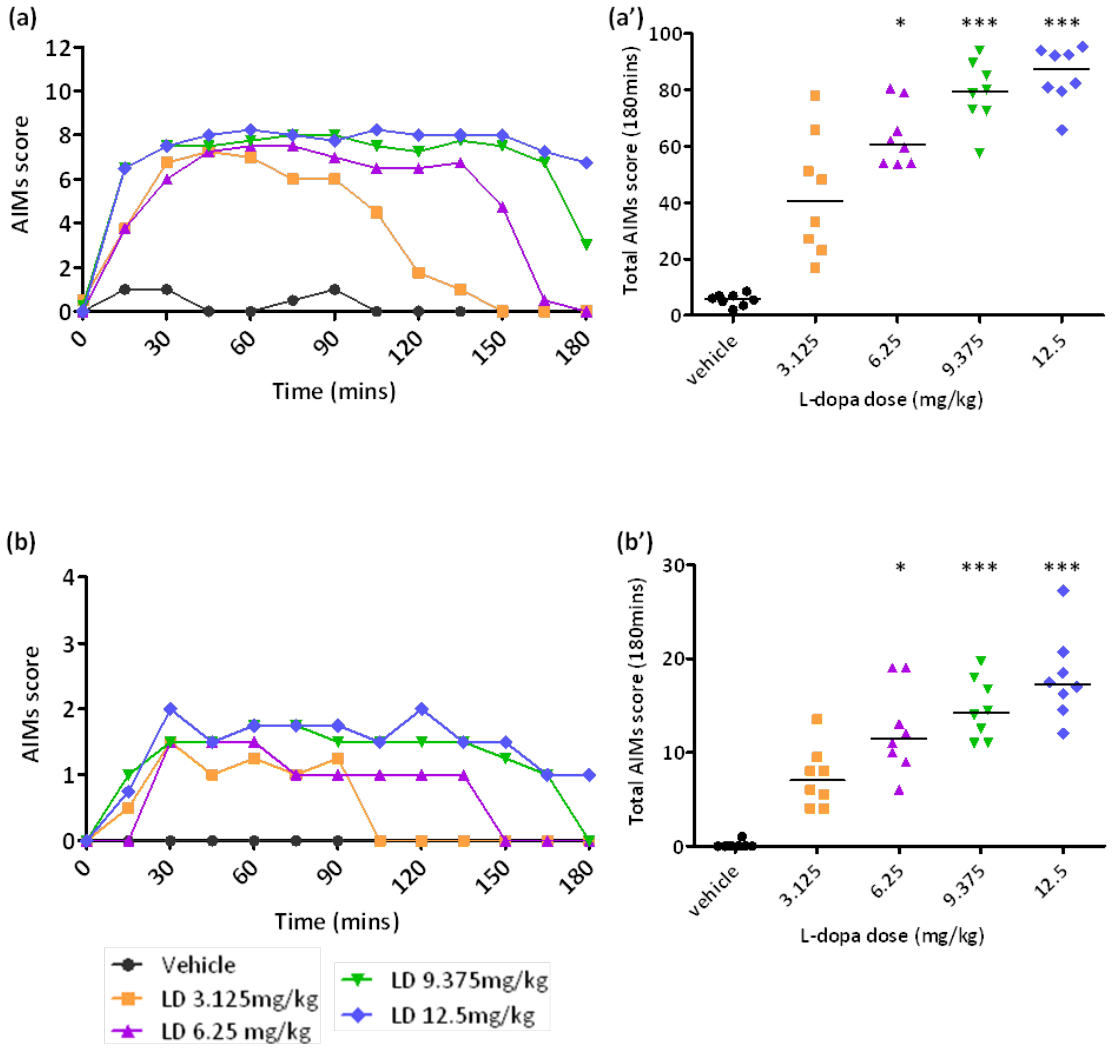

Figure 2-11 L-dopa dose-response ALO and locomotive AIMs data following L-dopa (LD) 3.125, 6.25, 9.375, or 12.5 mg/kg (plus benserazide 15 mg/kg i.p.) treatment. Data are presented as medians (n=8); Time-course of AIMs **(a)** ALO **(b)** locomotive, and also individual values; Total AIMs **(a')** ALO and **(b')** locomotive; *p<0.05, ***p<0.001 compared to vehicle treatment; Friedman's test followed by post hoc Dunn's test.

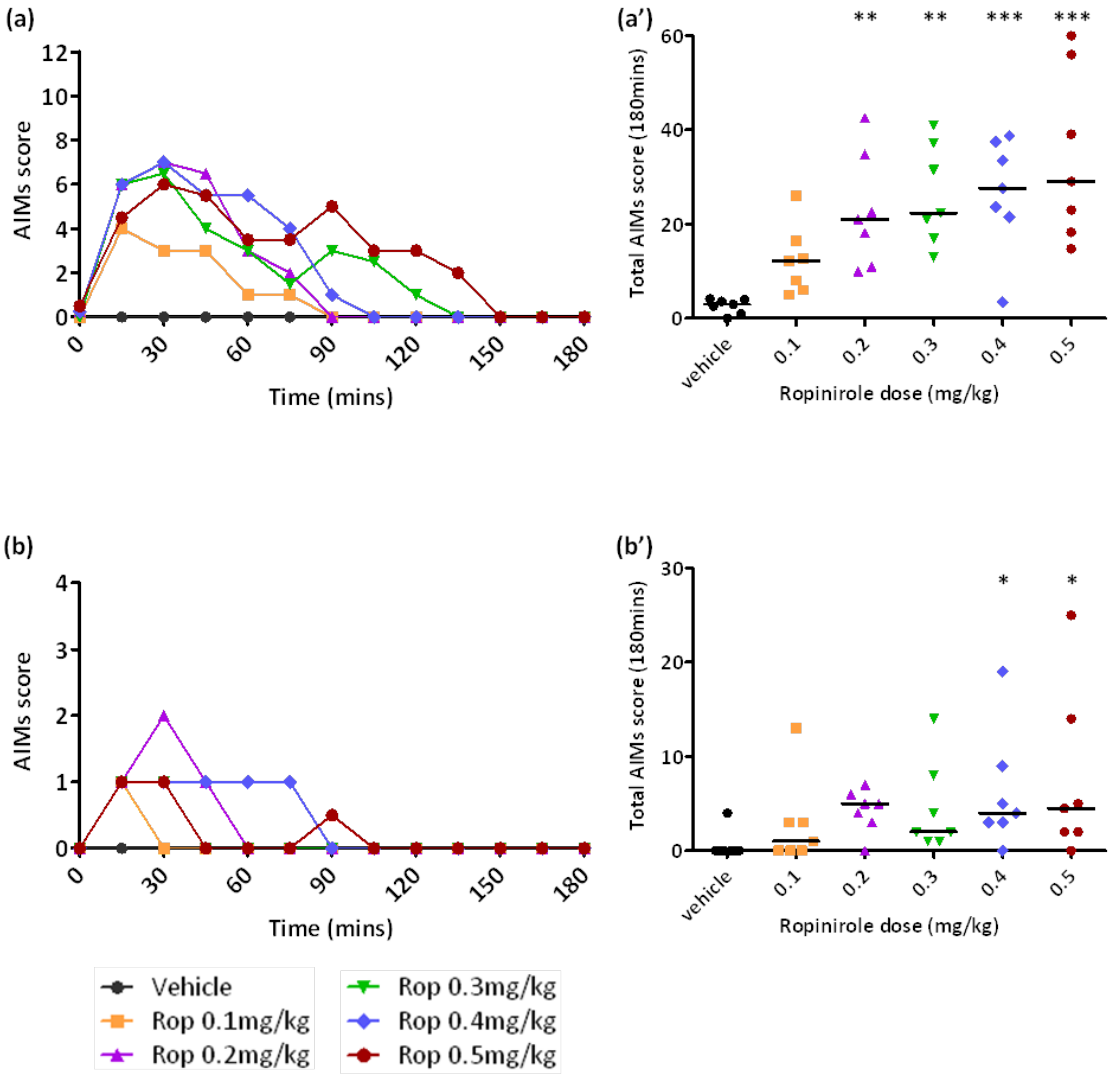

Figure 2-12 Ropinirole dose-response ALO and locomotive AIMs data following ropinirole (Rop) 0.1, 0.2, 0.3, 0.4 or 0.5 mg/kg i.p. treatment. Data are presented as medians (n=7); Time-course of AIMs **(a)** ALO and **(b)** locomotive, and also individual values; Total AIMs **(a')** ALO and **(b')** locomotive; ***p<0.001, **p<0.01 compared to vehicle treatment; Friedman's test followed by post hoc Dunn's test.

2.4 The MPTP-treated primate model:

2.4.1 Introduction

MPTP is a neurotoxin serendipitously found to cause a form of parkinsonism in humans indistinguishable in appearance from idiopathic PD (Langston *et al.*, 1983). Administration of MPTP to non-human primates induces a syndrome of parkinsonism closely resembling the human form. MPTP induces selective nigral dopaminergic cell loss via action of the metabolite 1-methyl-4-phenylpyridinium on mitochondria thus impairing energy production (Singer *et al.*, 1988). Dopaminergic agents commonly used in PD patients, including L-dopa and dopamine agonists, are able to reverse symptoms of motor disability seen in MPTP-treated primates. Priming of these animals with dopaminergic drugs leads to the characteristic display of dyskinesia with chorea and dystonia manifesting in a time-dependent manner (Pearce *et al.*, 1995). These uncontrollable motor movements are not disimilar to those seen in PD patients, indeed dyskinesia in primates is commonly assessed using scoring criteria with only minor modification from clinical ratings (Pearce *et al.*, 1995; Langston *et al.*, 2000). The model provides a very valuable measure for preclinical investigations, especially for translation of beneficial anti-dyskinetic agents into the clinic.

2.4.2 Animal husbandary

Adult common marmosets (*callithrax jacchus*) of either sex (n=15; 9 female and 6 male; weight 320 to 450 g; University of Manchester/Harlan UK) were used in this study. Animals were housed singly or in pairs at a temperature of 25±1 °C with 50 % relative humidity on a 12 h light/dark cycle. All animals were fed fresh fruit once daily and had *ad libitum* access to Mazuri food pellets (Mazuri Primate Diet, Special Diet Services Ltd., UK) and water. All animals were drug naïve prior to commencing this study. All experiments were carried out in accordance with Home Office regulations under the Animals (Scientific Procedures) Act 1986 and project license number 70/6345.

2.4.3 MPTP-lesion induction

Following an acclimatisation period of a minimum of 8 weeks marmosets were treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP-HCL; 2.0 mg/kg, in sterile saline 0.9 %, s.c.) (Sigma, UK/ Research Biochemicals International) daily for 5 consecutive days.

2.4.4 Post-MPTP care

For 10-16 weeks animals were hand-fed once-twice daily on a high protein/carbohydrate liquid diet (Marmoset jelly, Special Diet Services Ltd., UK and Complan, Complan Foods Ltd., UK) until they were able to feed independently and body weight had stabilised. Approximately 2 weeks after the end of treatment some animals developed a hyper-activity syndrome and home cages were padded with soft bedding materials to reduce risk of injury. Animals were monitored closely at all times to ensure recovery from the acute effects of the toxin and stabilisation of motor deficits.

2.4.5 Behavioural effects of MPTP administration

Within one week following the initiation of MPTP treatment animals developed symptoms akin to Parkinson's disease including bradykinesia, akinesia, rigidity and hypophonia. These were accompanied by a prominent loss of vocalisation, diminished blinking, incoordination and action tremor. A period of aphagia and adipsia lasting approximately 4 weeks also occurred. Gradual recovery followed and behavioural assessment indicated all animals had similar levels of motor deficits. The MPTP-treatment regimen, as described in section 2.4.3, routinely leads to a syndrome where behavioural deficiencies remain stable over several years (Jenner *et al.*, 1984).

2.5 The MPTP-treated primate model: Behavioural assessment

All animals were formally monitored for locomotor activity, motor disability and dyskinesia in specially designed aluminium test units (50 x 60 x 70 cm) with perspex doors (50 x 70 cm). All primate behavioural assessments were carried out between the hours of 7.00 am and 4.00 pm Animals were placed in individual test units and initially acclimatised for a 1 h period prior to drug treatment, and at 50-60 min a baseline score was taken. In addition to the three measurements outlined below general observational comments on the animal's behaviours were also recorded.

2.5.1 Priming for dyskinesia

Following recovery from the acute effects of MPTP a group of MPTP-treated primates (n=6) were treated daily with L-dopa methyl ester (12.5 mg/kg + carbidopa 12.5 mg/kg p.o.) for a period of 8 weeks, to establish stable dyskinesia levels as measured by behavioural rating scales. Animals were scored for dyskinesia (as shown in **Figure 2-13**) and motor disability, and locomotor activity data assessed, at baseline (day 0) and once-twice weekly thereon (see sections 2.5.2 - 2.5.4). Beyond the priming period animals were dosed weekly with L-dopa (12.5 mg/kg + carbidopa 12.5 mg/kg p.o.) to ensure a persistent dyskinetic response until subsequent studies commenced.

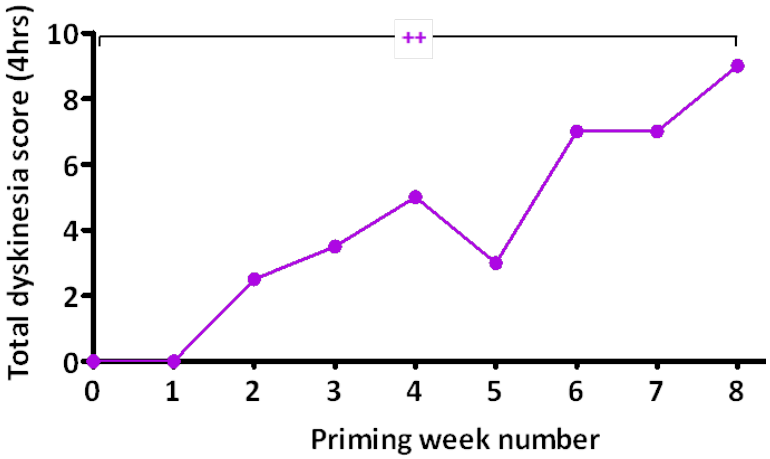

Figure 2-13 Dyskinesia development during L-dopa priming of MPTP-treated marmosets; animals (n=5) were treated with L-dopa methyl ester (12.5 mg/kg + carbidopa 12.5 mg/kg p.o.) on a daily basis for 8 weeks and scored for dyskinesia once-twice per week; total dyskinesia (for a 4 h scoring period) is presented as medians for each week and interquartile ranges were typically 6 dyskinesia units. ++$p<0.01$ for time; data were analysed by Friedman's test. (Data taken from Chapter 5).

2.5.2 Locomotor activity measurement

Test units were fitted with eight horizontally orientated infrared beams so as to detect movement of the marmosets. Three beams were localised to the cage floor, two along the perches (one per perch), one centrally on the front door and a further two directed perpendicular to each of the perches. Beam interruptions were relayed to computer software (DASYLab data acquisition system, laboratory version 11) throughout the test period and accumulated in 1, 10, and 30 min intervals. Data were set to download directly into an excel file for analysis.

2.5.3 Motor disability assessment

Motor disability was scored simultaneously to locomotor activity measurement through a one-way mirror during the last 10 min of consecutive 30 min time intervals. Initial baseline scores were made at 50-60 min after animals had been placed into the test units, at the end of the acclimatisation period. Scoring continued for a maximum 7 h period in total. Scores were based on the established motor disability rating scale (Pearce *et al.*, 1995), as detailed in **Table 2-3**, and the score for each period was taken as a total of the sub-scores ascribed to each of the individual criterion. A maximum motor disability score of 18 was possible, indicating a severely disabled marmoset. Typically an MPTP-treated marmoset will have a motor disability score of 10-14 for each assessment period without drug treatment. Meanwhile, naïve marmosets rarely score beyond 2 for motor disability.

Table 2-3 Motor disability scoring classification

Assessment criteria	Score
Alertness	0=normal, 1=reduced, 2=sleepy
Checking movements	0=present, 1=reduced, 2 =absent
Posture	0=normal, +1=abnormal trunk, +1=abnormal limbs, +1=abnormal tail, 4=grossly abnormal
Balance	0=normal, 1=impaired, 2=unstable, 3=spontaneous falling
Reactions	0=normal, 1=reduced, 2=slow, 3= absent
Vocalisation	0=normal, 1=reduced, 2=absent
Motility	0=normal, 1=bradykinesia, 2=akinesia

2.5.4 Dyskinesia assessment

Dyskinesia was rated at the same time as motor disability using an established dyskinesia rating scale (Pearce *et al.*, 1995) as summarised below (see **Table 2-4**). Chorea is characterised by rapid, dance-like movements predominantly of the limbs, and dystonia manifests as abnormal sustained posturing. Total dyskinesia takes into account both of these parameters. The score recorded for dystonia, chorea and also total dyskinesia is reflective of the quality and quantity of dyskinetic activity over the full 10 min observation period. Score increments of 0.5 were used where behaviour exhibited fell between assessment criteria.

Table 2-4 Dyskinesia scoring classification

Assessment criteria	Score
Absent	0
Mild fleeting and rare dyskinetic postures and movements	1
Moderate; more prominent abnormal movements but not significantly affecting normal behaviour	2
Marked; frequent and at times continuous dyskinesia affecting normal repertory of behaviour	3
Severe; virtually continuous dyskinesia disabling to animal and replacing normal behaviour	4

2.5.5 Establishing doses for L-Dopa and ropinirole

Introduction As for 6-OHDA-lesioned rats both L-dopa and dopamine agonists such as ropinirole lead to dyskinesia expression in MPTP-treated marmosets after long-term use. To investigate the potential effects of nNOS inhibitors on resulting dyskinetic behaviour it was necessary to determine the optimal doses of dopaminergic agents to be used in subsequent marmoset investigations.

Method A group of primed MPTP-treated marmosets (n=6) exhibiting stable dyskinesia (as described in section 2.5.1) was administered a range of doses of L-dopa methyl-ester or ropinirole to provide a dose-response curve for both drugs. A cross-over study with a modified latin-square design was employed including a minimum 2-day drug washout between challenges. Initially animals were treated with L-dopa methyl-ester (+ carbidopa 12.5 mg/kg) (3.125, 6.25, 9.375, 12.5 or 25.0 mg/kg p.o.) or sucrose (10 %, p.o.) at 2 ml/kg and assessed for locomotor activity, motor disability and dyskinesia in test units (described in section 2.5.2 - 2.5.4). In the next set of experiments animals were treated with ropinirole (+ domperidone 2 mg/kg) (0.1, 0.2, 0.3 or 0.4 mg/kg p.o.) or sucrose (10 %, p.o.) at 2 ml/kg and assessed as before. Behavioural time-course graphs and totals, calculated from the time courses by AUC (Graphpad Prism version 5.0) using the trapezoid method where each successive 30 min was labelled as a single time-bin, were plotted as medians (n=6). All total counts/score data were analysed by Friedman's test followed by post hoc Dunn's test.

Results As shown in **Figure 2-14** all doses of L-dopa administered to MPTP-treated marmosets increased locomotor activity with peak effects at approximately 90 min and gradually returning towards baseline levels thereafter in a dose-dependent manner. Locomotor activity increased in a dose-dependent manner as the L-dopa dose was increased up to 12.5 mg/kg above which there was a slight reduction in counts following L-dopa 25 mg/kg. The total locomotor activity was significantly increased compared to vehicle following L-dopa 12.5 and 25 mg/kg. L-dopa reversed motor disability in a dose-dependent manner peaking at 60 min after L-dopa administration. L-dopa increased motor disability reversal in terms of both severity and duration, as compared to vehicle treatment, although this effect did not reach statistical significance. Dyskinesia was only expressed at L-dopa doses of 6.25 mg/kg and above, with severity and duration increasing dose-dependently, reaching a peak at 60-90 min and stabilising for

30-60 min more and/or gradually returning towards baseline levels. Total dyskinesia increased with increasing L-dopa dose plateauing at 12.5 mg/kg. Both L-dopa doses of 12.5 and 25 mg/kg caused a significant increase in dyskinesia compared with vehicle treated animals.

As shown in **Figure 2-15** all ropinirole doses increased locomotor activity which peaked at 90-120 min after treatment, gradually returning to baseline levels by a maximum of 360 min in a dose-dependent time-frame. A significant increase in total locomotor activity was observed for ropinirole 0.2-0.4 mg/kg compared to vehicle treatment. The reversal of motor disability reached maximal levels by 60 min for ropinirole 0.2-0.4 mg/kg and 120 min for 0.1 mg/kg with a duration of effect increasing with dose. Total motor disability reversal was significantly increased at ropinirole doses of 0.2-0.4 mg/kg compared to vehicle. Animals expressed dyskinesia at all ropinirole doses peaking between 30-90 min after treatment and returning to baseline levels by 240 min. Peak dyskinesia scores also increased as a function of the ropinirole dose administered plateauing at 0.3 mg/kg. Ropinirole doses of 0.2-0.4 mg/kg resulted in significant levels of dyskinesia expression as compared to vehicle treatment.

Conclusion In primed MPTP-treated marmosets both L-dopa and ropinirole cause dyskinetic behaviour with a severity and time-course which increases with escalating dose. Compared to L-dopa, ropinirole appears to be longer acting although both drugs result in similar levels of dyskinesia at the doses employed. L-dopa 12.5 mg/kg and ropinirole 0.2 mg/kg were selected for further investigations based on these data.

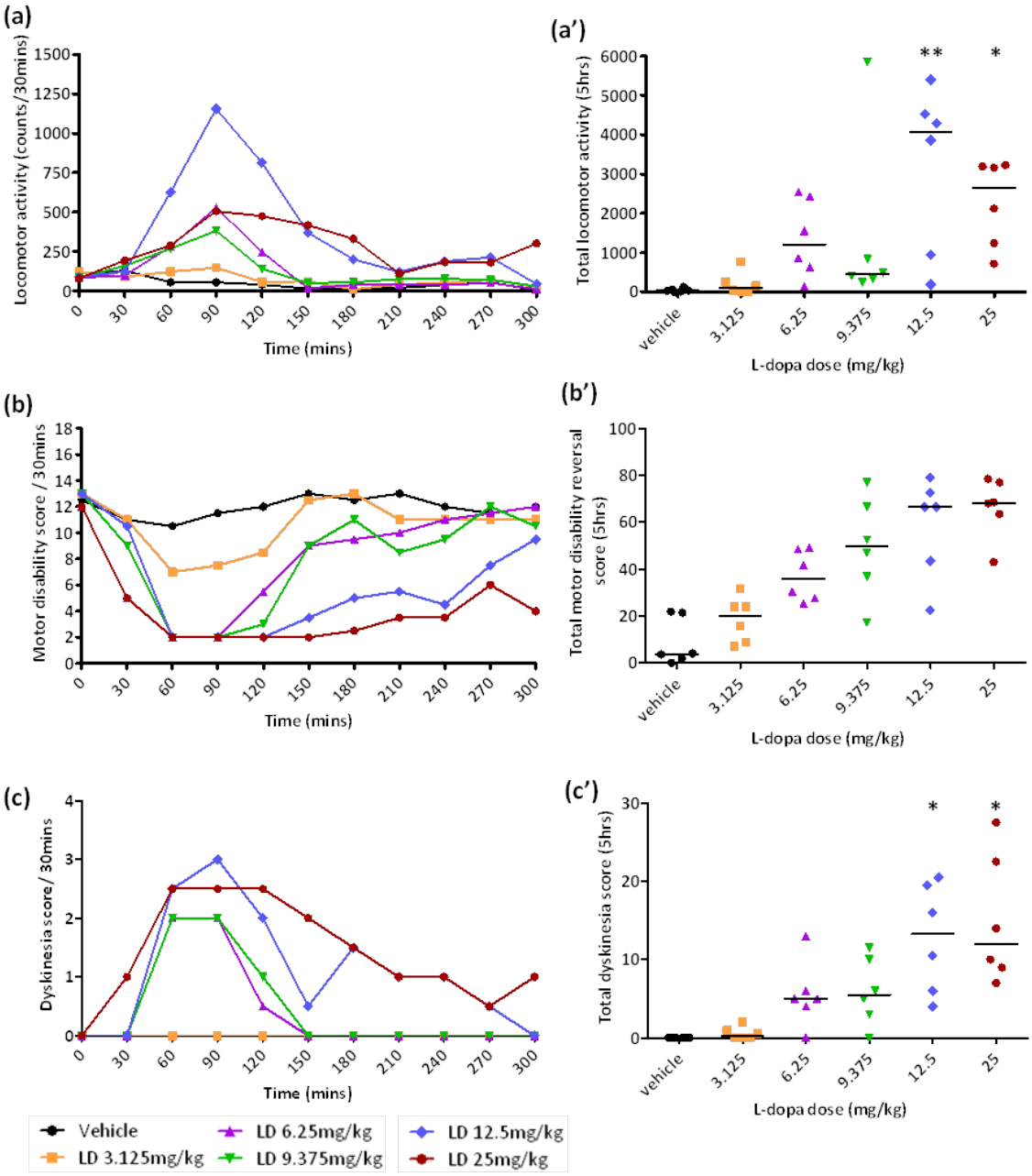

Figure 2-14 Behavioural assessment in MPTP-treated marmosets following L-dopa (LD) 3.125, 6.25, 9.375, or 12.5 or 25.0 mg/kg p.o. (+ carbidopa 12.5 mg/kg p.o.) treatment. Data are presented as medians (n=6); Time-course data for **(a)** Locomotor activity, **(b)** Motor Disability and **(c)** Dyskinesia, and also individual values; Total counts/score for **(a')** Locomotor activity, **(b')** Motor Disability and **(c')** Dyskinesia; *p<0.05 ,**p<0.01, compared to vehicle treatment; Friedman's test followed by Dunn's post hoc test.

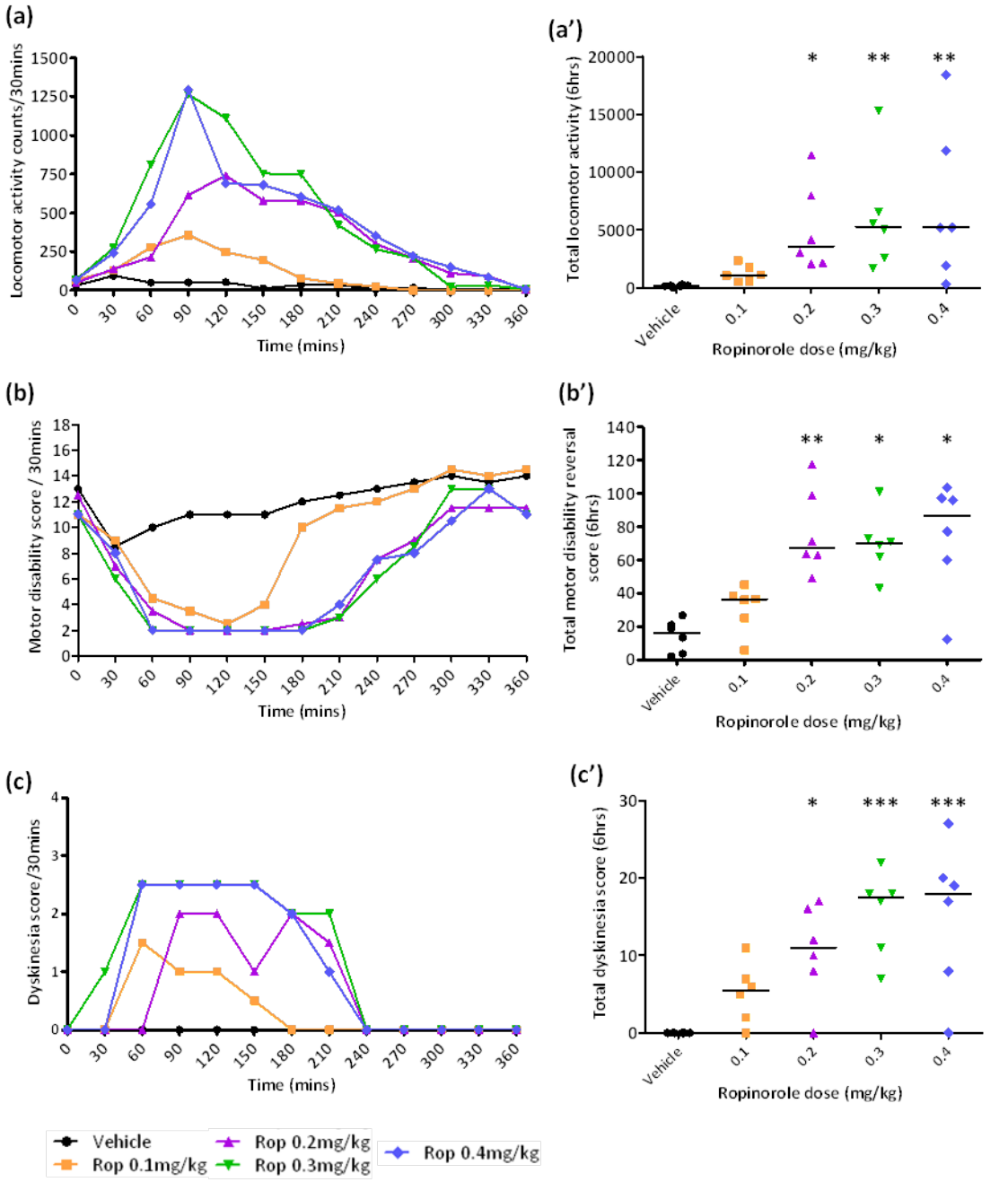

Figure 2-15 Behavioural assessment in MPTP-treated marmosets following ropinirole (Rop) 0.1, 0.2, 0.3 or 0.4 mg/kg p.o. (+ domperidone 2 mg/kg p.o.) treatment. Data are presented as medians (n=6); Time-course data for **(a)** Locomotor activity, **(b)** Motor Disability and **(c)** Dyskinesia, and also individual values; Total counts/score for; **(a')** Locomotor activity, **(b')** Motor Disability and **(c')** Dyskinesia; *p<0.05, **p<0.01, ***p<0.001 compared to vehicle treatment; Friedman's test followed by Dunn's post hoc

2.6 Biochemical Techniques

2.6.1 Radioenzymatic measurement of NOS activity in brain tissue

2.6.1.1 Collection of brain tissue

To determine activity levels of nNOS naïve male Wistar rats (250-275 g; Harlan) were used in *ex vivo* studies, and at the time point of interest post-dosing animals were killed by decapitation using a guillotene. Both the cerebellum and striatum were dissected out according to the atlas of Paxinos and Watson (1986) , snap frozen and stored at -70 ˚C until further use.

2.6.1.2 nNOS radioactivity assay

nNOS activity was measured by monitoring radioactivity levels following the enzymatic conversion of [^{3}H]-arginine to [^{3}H]-citrulline as described by Bredt and Snyder (1989). The radioenzymatic assay is described below.

The NOS assay was performed in a 96 well-plate and all reagents apart from the reaction mixture prepared in advance (**Table 2-5**) and kept on ice until use. Brain tissue samples were weighed and 5 x volume of 1 x homogenisation buffer (**Table 2-5**) containing Protease inhibitor cocktail set III (1:20 dilution) was added. Tissue samples (plus homogenisation mix) were then homogenised, using a cordless motor pellet pestle homogeniser (Kontes), and centrifuged (for 1 h at 1000 g at 4 °C). Supernatant was transferred into newly labelled eppindorfs and aliquots (20 µl) were transferred into a 96 well plate in triplicate. The same volume of boiled supernatant (10 min at 100 °C) was used as a control. Next the reaction mixture (30 µl) (as described in **Table 2-5**) was added to each well and the plate placed in an incubator at 30 °C while gently shaking.

After 1 h of incubation the reaction was terminated by adding 50 µl stop buffer to each well. Additionally 100 µl of equilibrated resin (Biorad AG 50W Cation Resin, see **Table 2-5**) was added to each sample and subsequently incubated for 45 min at 30 °C while gently shaking. Then the reactions were pipetted into Multiscreen HTS DV 0.65 µM filtration plates (Millipore) and filtered using a chemical duty vacuum pump (Millipore). The [^{3}H]-citrulline, being ionically neutral, flowed through the filter whilst the positively charged [^{3}H]-arginine stayed bound to the resin. Finally eluate (10 µl/well) was transferred into a 96-well microplate containing 100 µl Scintillant (Ultima Gold, Perkin-Elmer) per well. NOS activity was quantified by using a Wallac Beta-liquid scintillation counter yielding measurements in counts per minute (cpm) (40 % efficiency).

Table 2-5 Reagents for radioenzymatic NOS assay

Reagent	Components
10 x Homogenisation Buffer	250 mM Tris-HCL (pH 7.4) 10 mM ethylenediaminetetraacetic acid (EDTA) disodium salt 10 mM ethyleneglycol-bis(β -aminoethyl ether)-N,N,N´,N´-tetraacetic acid (EGTA)
Reaction buffer	50 mM Tris-HCl (pH 7.4) 6 µM tetrahydrobiopterin (BH_4) 2 µM flavin adenine dinucleotide (FAD) 2 µM flavin adenine mononucleotide (FMN)
Stop buffer	50 mM *N*-2-hydroxyethylpiperazine- *N´*-2-ethanesulfonic acid (HEPES) (pH 5.5) 5 mM EDTA disodium salt
Equilibrated Resin	12.5 % cation resin (w/v) suspended in sodium hydroxide for 1hr at RT, wash with dH_2O to pH7-10. Eqilibrate with 10mM HEPES to pH 6.5
Reaction mixture	1 x reaction buffer (as above) 6 mM $CaCl_2$ (3.6 mM/well) 10 mM reduced nicotinamide adenine dinucleotide phosphate (β-NADPH) 16.7 µM L-[2,3,4-^{3}H] arginine monohydrochloride

2.6.1.3 Protein content determination

Protein content of tissue homogenate was measured using a NanoDrop spectrophotometer (ND-1000) in conjunction with ND-1000 3.5.1 software. Samples of homogenate (2 µl) were loaded on to the lower measurement pedestal of the NanoDrop and light absorbance measured at 280 nm (A280) (See **Figure 2-16**); samples were analysed in triplicate. Measurements of absorbance are directly proportional to protein concentration of BSA standards (**Figure 2-17**).

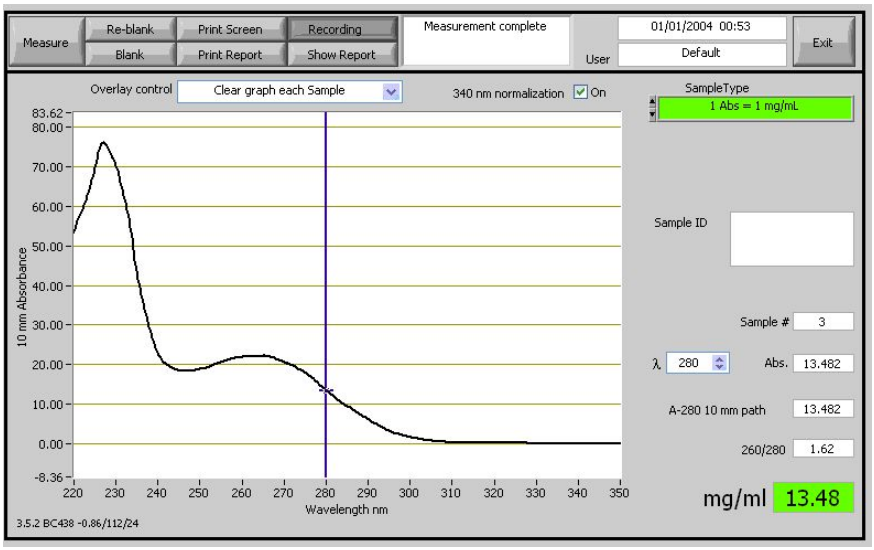

Figure 2-16 Example of protein measurement using the NanoDrop spectrophotometer. Protein content of cerebellar and striatal samples were determined in triplicate using this equipment. Light absorbance was measured for the sample and compared to internal standards.

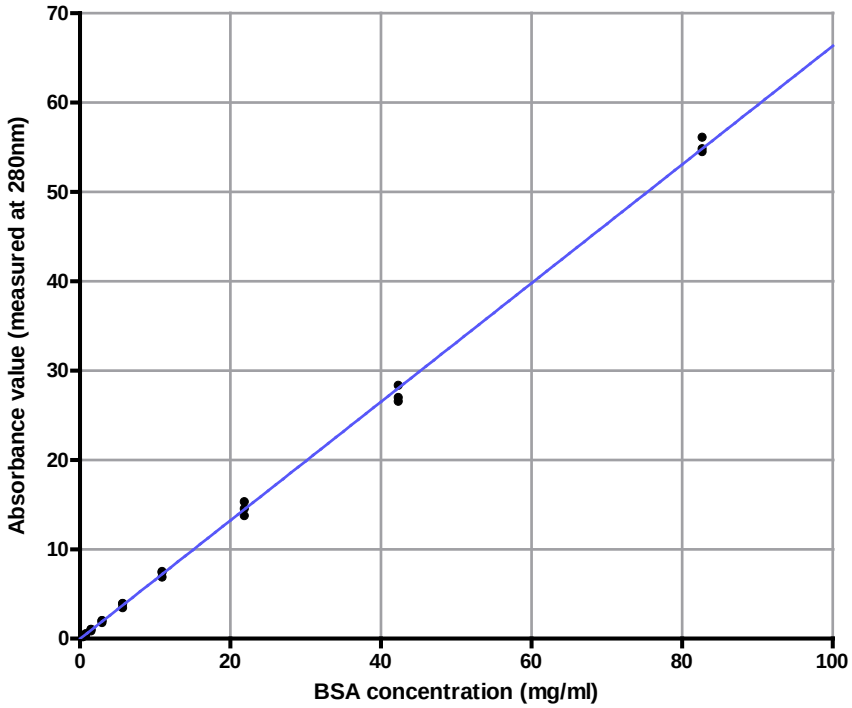

Figure 2-17 Protein content standard curve from Nanodrop. Protein standards were prepared in triplicate and the absorbance of 2 µl BSA samples read at 280 nm; the graph demonstrates the linear relationship between absorbance and protein concentration.

2.6.1.4 Data and statistical analysis

To evaluate nNOS activity the average cpm from the boiled control was subtracted from the average cpm of the tissue sample. Dividing by the average tissue sample protein measurement (mg/ml) obtained from the Nanodrop (as described in section 2.6.1.3) this cpm figure was converted into cpm per mg of protein. Statistical analysis was carried out using a one-way ANOVA followed by *post hoc* Newman-Keuls test. Results as exemplified in **Figure 2-18a** were expressed as L-[3H]-citrulline formation (cpm per mg protein; specific activity is 47.9 cpm/fmol) . Calcium-independent NOS activity accounts for less than 3 % of the overall activity as measured when samples were assayed using a reaction mixture where $CaCl_2$ was replaced by deionised water (**Figure 2-18 b**). iNOS activity is therefore negligible in comparison to calcium-dependent NOS activity (in the CNS this is predominantly accounted for by nNOS).

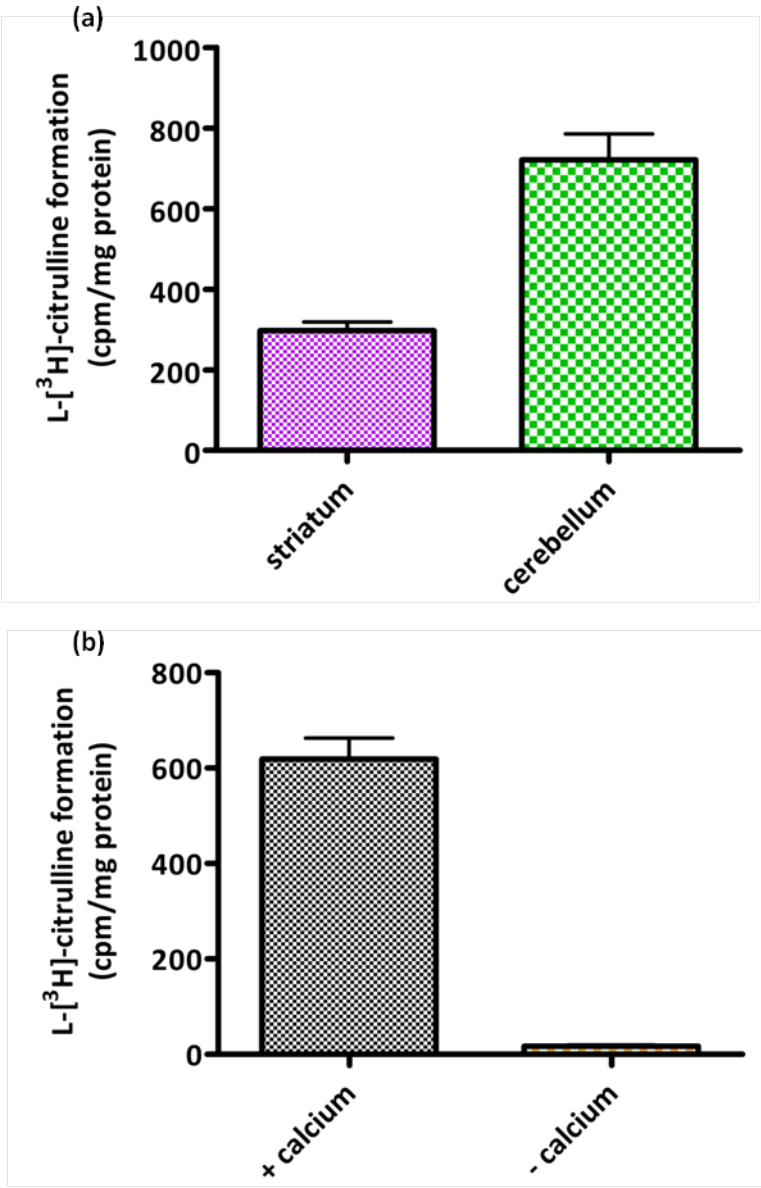

Figure 2-18 Example of radioenzymatic measurement of nNOS activity in brain homogenates. Samples taken from naïve rats treated with saline (0.9 %) and culled by decapitation at 1hr **(a)** Striatal and cerebellar samples assayed following standard protocol (n=4), **(b)** Cerebellar samples assayed with (+) (standard protocol) and without (—) $CaCl_2$ in the reaction mixture (n=3) Data are presented as mean ± SEM.

2.6.2 Determination of tyrosine hydroxylase

In order to confirm the lesion size in 6-OHDA rats, immunohistochemical analysis of tyrosine hydroxylase (TH) positive cells was used to quantify dopamine neurones in the substantia nigra pars compacta.

2.6.2.1 Collection of brain tissue

At the conclusion of behavioural studies (see section 2.3) 6-OHDA-lesioned rats were administered a lethal dose of sodium pentobarbitol (100 mg/kg ip). Once animals had lost reflex reactions the thorax was opened to expose the heart and perfused with 100 ml of ice-cold 0.1 M phosphate buffered saline (PBS; pH 7.4) followed by 100 ml ice-cold paraformaldehyde (PFA; 4 % in 0.1 M PBS). Animal were then decapitated and brains dissected from the skull and transferred to 4 % PFA for post-fixation at 4 ˚C. After 2-3 days brains were moved to 30 % sucrose (with 0.05 % sodium azide) for cryoprotection and stored at 4 ˚C until they sunk. Brains were maintained in these conditions until cutting commenced.

2.6.2.2 Processing of brain tissue

Brains were cut on a Leica freezing microtome (SM2000R) and collected as coronal sections of 30 µm thickness in 24-well plates. The cerebellum was initially removed with a blade and the remaining brain tissue mounted on a cold metal stage with Cryo-M-bed, embedding compound. The brain was cooled to -30 ˚C, using cryospray to assist, and adjoining sections of tissue throughout the level of the substantia nigra were collected in series. Sections were stored, 4 per well, in 0.1 M PBS (with 0.05 % sodium azide) at 4 ˚C until further use.

2.6.2.3 Immunohistochemistry

In preparation for TH staining, sections were incubated in 1 % H_2O_2 in 0.1 M PBS for 35 min to block endogenous peroxidase activity. They were then permeabilised by washing twice for 10 min in 0.05 % Triton-X in 0.1 M PBS (PBS-TX). Sections were blocked for 1 h with 20 % normal goat serum (NGS) in PBS-TX followed by incubation in primary antibody (rabbit TH diluted 1:500, in 2 % NGS in PBS-TX) overnight at room temperature.

After primary antibody incubation sections were washed twice for 10 min in PBS-TX and subsequently incubated with biotinylated secondary antibody (anti-rabbit TH diluted 1:200, in PBS-TX) for 1.5 h at room temperature. Next, sections were again washed twice for 10 min in PBS-TX and incubated for 1 h with Avidin biotin complex (ABC), as prepared during the secondary antibody incubation. Sections were then washed twice for 10 min in Tris-HCL buffer (0.1 M; pH 7.4) before incubating with DAB (0.05 % in Tris-HCL buffer) plus H_2O_2 (0.03 %) for 30-60 seconds. The reaction was terminated by transferring sections through a Tris-HCl buffer rinse and two distilled water rinses. Sections were then mounted onto polylysine coated slides (VWR International) and left to dry overnight.

The next day sections were rinsed in distilled water and dehydrated through an escalating series of ethanol solutions (2 min each in 70, 98 & 100 %). Sections were subsequently delipidated in histoclear for 10 min and cover slipped using DePeX mounting medium. Slides were left to dry overnight before viewing under the microscope.

2.6.2.4 Cell counts

TH positive cells stained by immunohistochemistry as described in 2.6.2.3 were counted using an Olympus (BX61) Light Microscope. For each coronal brain section all stained cells appearing in the SNpc at the level of the third nerve were included in the count which was conducted on both lesioned (left) and non-lesioned (right) sides to allow comparison (see **Figure 2-19**). An Olympus (DP70) digital camera in combination with Cell D Analysis imaging software were used to capture photographs of sections used for counting.

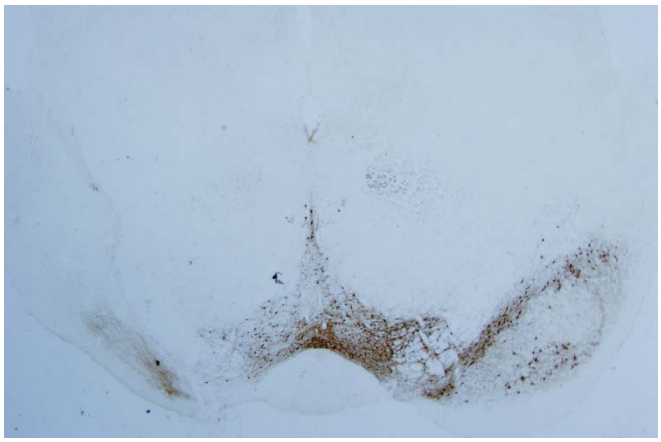

Figure 2-19 TH Immunohistochemistry of 6-OHDA-lesioned rat brain section; substantia nigra shown at the level of third nerve following a lesion in the medial forebrain bundle. Brown stained areas depict dopaminergic cell bodies or projections. Image shows the lesioned side on left and non-lesioned side on right.

2.7 General materials used

Table 2-6 List of drugs

Item	Supplier
L-DOPA methyl ester	Sigma
Benserazide	Sigma
Carbidopa	Sigma
Ropinirole hydrochloride	Sigma
7-Nitroindazole (7-NI)	Tocris Bioscience, Bristol, UK
ARR17477 HCL salt	Mercachem, The Netherlands / WuxiAppTec, China
(+)-MK-801 hydrogen maleate	Sigma
(+)-8-Hydroxy-2-(dipropylamino)tetralin(8 OHDPAT)	Tocris Bioscience, Bristol, UK
Amantadine hydrochloride	Sigma

Table 2-7 List of chemicals and reagents

Item	Supplier
Avidin biotin complex (ABC)	Vector Laboratories, Peterborough, UK
Biotinylated goat anti-rabbit IgG	Vector Laboratories, Peterborough, UK
Calcium chloride	Applichem, Biochemica
Cryo-M-bed, embedding compound	Bright Instrument Co Ltd, Cambridgeshire, UK
DAB kit	Vector Laboratories, Peterborough, UK
DePeX mounting medium	BDH, VWR International, Lutterworth, UK
Cryospray	Bright Instrument Co Ltd, Cambridgeshire, UK
EMLA cream	AstraZeneca, UK
Ethanol	Fisher Scientific, Leicestershire, UK
Goat serum	Vector Laboratories, Peterborough, UK
Histoclear	VWR International, Lutterworth, UK
Homogenisation Buffer (x10)	Stratagene, Leicester, UK
Isofluorane	Abbott Laboratories Ltd, Berkshire, UK
L-[2,3,4-^{3}H] Arginine Monohydrochloride	GE Healthcare, Amersham, UK / Perkin-Elmer, Beaconsfield, UK
Protease Inhibitor cocktail set III	Calbiochem, Nottingham, UK
Resin AG50W-X8 (100-200 dry mesh, 106-250 µm wet bead)	Bio-Rad Laboratories Ltd. Hertfordshire, UK
Rimadyl	Pfizer Animal Health, Kent, UK
Sodium Chloride	BDH, VWR International, Lutterworth, UK

Sodium Pentobarbitol (Euthanal)	Merial, Dundee, UK
Tyrosine hydroxylase antibody (rabbit)	Pel-Freez, USA
Tris (hydroxylmethyl) methylammonium chloride (Tris-HCL)	BDH, VWR International, Lutterworth, UK
Ultima gold scintillant	Perkin-Elmer, Beaconsfield, UK

Chemicals and reagents not listed were acquired from Sigma-Aldrich, Dorset, UK

Table 2-8 List of equipment and consumables

Item	Supplier
12- and 24-Well Plates	Nunc, Roskilde, Denmark
AIMs cages	Workshop, King's College London
Centrifuge	Meadowrose Scientific Ltd., Oxfordshire, UK
Chemical duty vacuum/pressure pump 220 vol (WP6122050)	Millipore, Livingston, UK
Cover slips	VWR International, Lutterworth, UK
DASYLab data acquisition system, laboratory version 11	Adept Scientific plc, Herts, UK
Ethovision software	Tracksys Ltd., Nottingham, UK
Hamilton syringe 10 µl (701RN)	Hamilton Company, Nevada, USA
Micro-injector syringe pump 'Nanomite'	Harvard Apparatus, Kent, UK
Microplate, Corning 96-well plate	Fisher Scientific, Leicestershire, UK
Microscope (BX61)	Olympus, Essex, UK
Microscope digital camera (DP70)	Olympus, Essex, UK
Microtome SM 2000R	Leica Microsystems, Milton Keynes, UK
Motor pellet pestle homogeniser	Fisher Scientific, Leicestershire, UK
Multiscreen HTS DV 0.65 µM filter plates	Millipore, Livingston, UK
Nanodrop ND-1000 Spectrophotometer	Labtech International, East Sussex, UK
Pipette tips	Starlab Ltd., Milton Keynes, UK
Polysine-coated slides	VWR International, Lutterworth, UK
Primate behavioural test units	Workshop, King's College London
Rotometry arenas	Workshop, King's College London
Stereotaxic Frame	Kopf Instruments
Sutures	Ethicon, Johnson & Johnson
Swabs (70 % isopropyl alcohol)	Uhs, Enfield, UK
Tracksys Video monitoring Software and Equipment	Tracksys Ltd., Nottingham, UK
Wallac MicroBeta Liquid scintillation counter	Perkin Elmer, Beaconsfield, UK
Wide orifice filter pipet tips	VWR international, Lutterworth, UK

Chapter 3： The effects of nNOS inhibitor treatment on expression of AIMs in 6-OHDA-lesioned rats

3.1 Introduction

Dyskinesia presents a significant clinical problem in PD surfacing where long-term dopaminergic medication becomes necessary to reverse the characteristic motor symptoms of the disease. Indeed PD patients have a 40 % likelihood of developing dyskinesia following 4-6 years of L-dopa therapy, although it is notable that their levels are not troublesome in all cases (Ahlskog & Muenter, 2001). To date the only agent which appears to reduce the severity of established dyskinesia, without worsening of parkinsonian symptoms, is amantadine (Luginger *et al.*, 2000; Snow *et al.*, 2000), but it is only effective in a small proportion of patients and is poorly tolerated with benefits often transient (Stocchi *et al.*, 2008). Importantly the beneficial effects of amantadine suggest that it is possible to attenuate dyskinesia once it has emerged but there is a clear requirement for more effective anti-dyskinetic interventions.

Targeting the nitrergic sytem has received little attention as a therapy option for dyskinesia despite the key role of nitric oxide as a neuromodulator in the CNS. There exists a strong rationale for investigating nNOS inhibitors in the management of dyskinesia, as discussed in further detail in Chapter 1 (see section 1.4). This opportunity is supported by changes in nitric oxide, nNOS and nNOS mRNA in PD patients and animal models of PD (Eve *et al.*, 1998; Gatto *et al.*, 2000).

The nNOS inhibitor 7-NI has been utilised in a range of *in vivo* studies to date, and therefore provides an opportune probe, although its selectivity for nNOS over other NOS isoforms is arguable (Moore *et al.*, 1993a; Zagvazdin *et al.*, 1996). The more recently discovered AstraZeneca compound ARR17477 (Zhang *et al.*, 1996) is described as a comparatively potent and selective inhibitor of nNOS relative to other NOS isoforms, and whilst there are limited experimental data examining its use *in vivo* especially concerning routes of administration beyond i.v. (O'Neill *et al.*, 2000; Reif *et al.*, 2000), further characterisation would be a worthwhile endeavour owing to its favourable properties. By employing both of these compounds as tools, it is logical that their combined advantages could lead to findings regarding the specific effects of nNOS inhibition.

Furthermore, the established 6-OHDA-lesioned rat offers a validated and accessible model in which to test for the potential benefits of nNOS inhibitors in PD. Following chronic dosing with L-dopa, animals rapidly develop abnormal movements which can be rated by a trained observer. The abnormal involuntary movement (AIMs) model as established by Cenci and colleagues (1998) has been extensively characterised in the published literature to date and is widely used for testing therapeutic agents in PD (Lundblad *et al.*, 2002; Dekundy *et al.*, 2007; Dupre *et al.*, 2008). Although the model may not be entirely representative of the progressive nature of dyskinesia manifestation in man, drugs reported to reduce dyskinesia in the clinic such as amantadine and clozapine have been confirmed effective in this model (Lundblad *et al.*, 2002; Dekundy *et al.*, 2007). Thus the 6-OHDA-lesioned rat was selected for studies to investigate dyskinesia via AIMs assessment.

Once dyskinesia manifests, there is some evidence in PD patients, MPTP-treated primates and 6-OHDA-lesioned rats suggesting its expression tends to be lower with dopamine agonists as opposed to L-dopa (Kapoon *et al.*, 1989; Facca & Sanchez-Ramos, 1996; Hadj Tahar *et al.*, 2000; Jackson *et al.*, 2007; Papathanou *et al.*, 2011). In severe cases, patients may be switched to dopamine agonist monotherapy in an attempt to alleviate the dyskinesia or more commonly a combination of L-dopa and dopamine agonists may allow the L-dopa dose to be reduced (Rascol *et al.*, 2002b). Anti-dyskinetic agents may therefore have differing degrees of effect on the control of motor abnormalities, dependent upon whether L-dopa or a dopamine agonist e.g. ropinirole is being administered. Thus the chosen dopaminergic drug is an important factor to consider when assessing the potential of any anti-dyskinetic adjunct therapy.

Thus the purpose of these studies was to further explore the link between NO and dyskinesia expression following dopaminergic treatment in PD. To this end it was necessary to extend the characterisation of ARR17477 and 7-NI inhibition of neuronal NOS to then enable investigation into the expression of dyskinesia in the 6-OHDA-lesioned rat model of PD.

3.1.1 Hypothesis

It is hypothesised that inhibibition of nNOS will reduce the expression of established dyskinesia induced by dopaminergic drugs in 6-OHDA-lesioned rats.

3.1.2 Aims

The aim of these studies was to determine whether nNOS inhibitors can reduce the expression of dyskinesia following acute challenges of dopaminergic treatment in L-dopa primed-6-OHDA-lesioned rats, and specifically to investigate;

1. The effect of the selective nNOS inhibitors ARR17477 and 7-NI on nNOS activity *ex vivo* following a range of doses.

2. The acute behavioural effect of ARR17477 and 7-NI administered in combination with L-dopa on dyskinesia expression in L-dopa-primed 6-OHDA-lesioned rats.

3. The acute behavioural effect of ARR17477 and 7-NI administered in combination with ropinirole on dyskinesia expression in L-dopa-primed 6-OHDA-lesioned rats.

3.2 Materials and methods

3.2.1 Introduction

Initially *ex vivo* studies were carried out in order to establish the dose of nNOS inhibitors for *in vivo* studies. Secondly the effect of nNOS inhibitors was investigated in rats with a 6-OHDA lesion and primed to express AIMS (dyskinesia), by chronic dosing with L-dopa. Acute studies, with the pre-selected doses of nNOS inhibitors were carried out to determine the effect on both L-dopa- and ropinirole-induced AIMs. The methods for these studies are described below.

3.2.2 Animals

For all studies male Wistar rats (200-250 g; Harlan, UK or B & K, UK) were housed 2-3 per cage in the Biological Service Unit, King's College London. Room temperatures were maintained at 19-21 ˚C at 55 % humidity with a 12 h light-dark cycle and animals had free access to pelleted food and water, as described in section 2.2.2. Experiments were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986 under Home Office project licence no. 70/6019 or 70/6898.

3.2.3 Ex vivo NOS assay

To determine the effect of the nNOS inhibitors ARR17477 and 7-NI on nNOS activity in rat brain, enzyme activity was assessed in rats by measuring the conversion of L-arginine to L-citrulline *ex vivo* following administration of a range of doses.

3.2.3.1 Establishing nNOS inhibitor doses for acute AIMs studies

The doses of the nNOS inhibitors ARR17477 and 7NI to be used in these studies were determined in experiments measuring the inhibition of nNOS activity *ex vivo* following systemic administration.

3.2.3.1.1 Determination of ARR17477 doses

Naïve male Wistar rats (n=4/group; 200-250 g) were treated with ARR17477 (3, 6 or 12 mg/kg in 0.9 % saline s.c.) or vehicle (saline 0.9 %; 1 ml/kg s.c.). These doses were chosen based on published studies (O'Neill *et al.*, 2000; Reif *et al.*, 2000) and preliminary data from our laboratory showing that ARR17477 (10 mg/kg i.p.) reduced striatal nNOS activity by 67 % at 1 h.

Animals were culled at one hour after ARR17477 treatment by decapitation. Both the cerebellum and striatum were dissected out (as described in 2.6.1.1), snap frozen and stored at -70 ˚C for measurement of nNOS activity by radioenzymatic assay.

3.2.3.1.2 Determination of 7NI doses

Naïve male Wistar rats (n=4/group; 200-250 g) were treated with 7NI (12.5, 25 or 50 mg/kg dissolved in DMSO:saline 0.9 %, 50:50, i.p.) or vehicle (DMSO:saline 0.9 %, 50:50 i.p.). These doses were selected on the basis of dose ranges utilised in published literature (Mackenzie *et al.*, 1994; Moore & Bland-Ward, 1996; Bush & Pollack, 2000).

Animals were culled after 1 h by decapitation. Both the cerebellum and striatum were dissected out (as described in section 2.6.1.1), snap frozen and stored at -70 ˚C for measurement of nNOS activity by radioenzymatic assay.

3.2.3.2 Radioenzymatic measurement of NOS activity

nNOS activity was determined in brain homogenates by measuring enzymatic conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline as fully described in section 2.6.1. Briefly homogenised tissue samples were centrifuged (as detailed in section 2.6.1.2), and supernatants transferred to a 96-well plate in triplicate. Boiled supernatant was used as a negative control. A pre-prepared reaction mixture containing L-[2,3,4-^{3}H] arginine monohydrochloride (1 mCi/ml; 16.7 µM) and $CaCl_2$ (6 mM) was added (30 µl/well) and the plates incubated for 1 h at 30 °C. The reaction was terminated and the reaction mixture further processed to measure L-[^{3}H]-citrulline formation, by the addition of resin and subsequent filtration of the samples as described in section 2.6.1.2. L-[^{3}H]-citrulline was determineded in a Beta-liquid scintiallation counter (see section 2.6.1.2) whilst protein content of samples was quantifieded with a NanoDrop spectrophotometer (see section 2.6.1.3). Data were analysed as described in section 2.6.1.4 expressing nNOS activity as L-[^{3}H]-citrulline formation per mg of protein per hour.

3.2.3.2.1 ARR17477 results

ARR17477 (6 and 12 mg/kg) significantly reduced nNOS activity by 41 % and 56 % respectively in the cerebellum (**Figure 3-1a**). Similarly, ARR17477 (3, 6 and 12 mg/kg) significantly reduced nNOS activity in a dose-dependent manner ranging from 34-60 % inhibition in striatal tissue (**Figure 3-1b**) and were hence chosen for further study.

3.2.3.2.2 7-NI results

7-NI (12.5, 25 and 50 mg/kg) significantly reduced nNOS activity by 30 % at the lowest dose administered, 58 % at 25 mg/kg and 49 % at 50 mg/kg in cerebellar tissue (**Figure 3-2a**). 7-NI (12.5, 25 and 50 mg/kg) also significantly reduced nNOS activity in the striatum by 41-43 % at the lower two doses and 29 % at the highest drug dose (**Figure 3-2b**). All three doses of 7-NI were therefore used in subsequent studies.

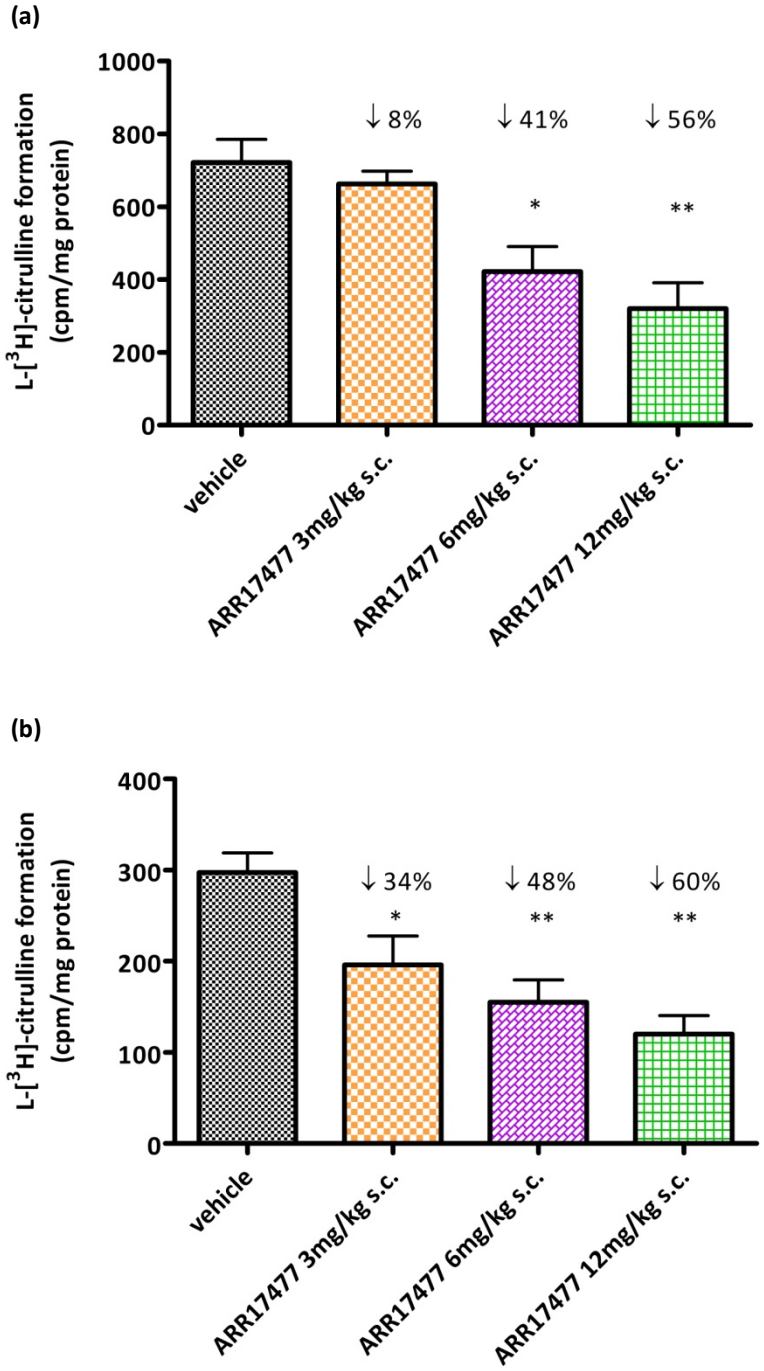

Figure 3-1 Radioenzymatic measurement of the effect of ARR17477 (3, 6 or 12 mg/kg s.c.) on nNOS activity in cerebellum and striatum. Naïve male Wistar rats were treated with ARR17477 or vehicle (saline 0.9 % s.c.) and culled by decapitation at 1hr. Tissue samples were obtained from **(a)** cerebellum and **(b)** striatum. nNOS activity is displayed as L-[3H]-citrulline formation (cpm/mg protein); Data are presented as means ± SEM (n=4/group); *P<0.05, ** P<0.01 compared to vehicle treatment. Data were analysed by one-way ANOVA followed by Newman-Keuls test.

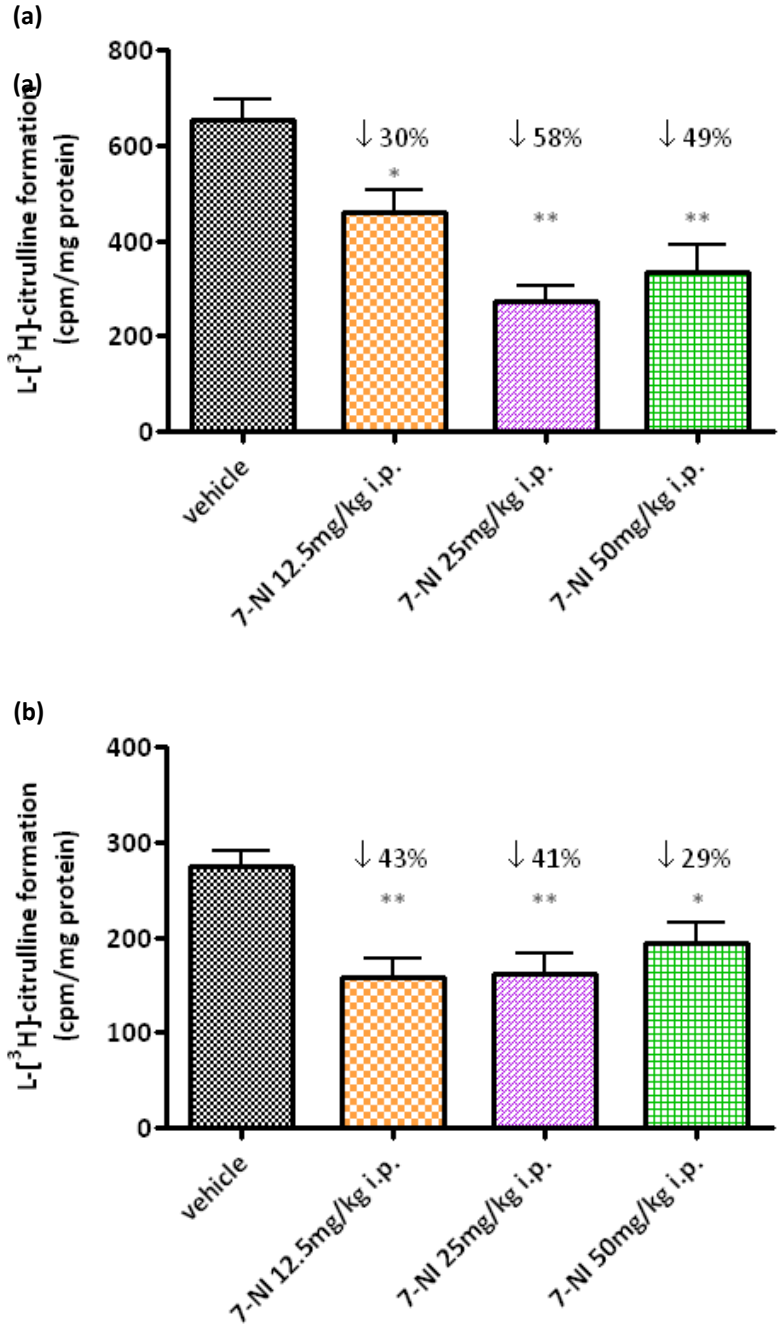

Figure 3-2 Radioenzymatic measurement of the effect of 7-NI (12.5, 25 or 50 mg/kg i.p.) on nNOS activity in cerebellum and striatum. Naïve male Wistar rats were treated with 7-NI or vehicle (DMSO:saline 0.9 %, 50:50 i.p.) and culled by decapitation at 1hr. Tissue samples were obtained from **(a)** cerebellum and **(b)** striatum; nNOS activity is displayed as L-[3H]-citrulline formation (cpm/mg protein); Data are presented as means ± SEM (n=4/group); *P<0.05, ** P<0.01 compared to vehicle treatment. Data were analysed by one-way ANOVA followed by Newman-Keuls test.

3.2.4 Assessment of the effect of nNOS inhibition on AIMs in L-dopa primed 6-OHDA-lesioned rats

3.2.4.1 Unilateral 6-OHDA lesion

Male Wistar rats (see section 3.2.2) were unilaterally lesioned with 6-OHDA under general anaesthesia (as described fully in section 2.2.3) using standard stereotaxic techniques. In brief, 6-OHDA hydrochloride (8 µg free base in 4µl 0.9 % saline containing 0.05 % ascorbic acid) was injected at a rate of 1 µl per minute with a Hamilton syringe and 26S gauge needle into the left MFB (according to the coordinates of Paxinos and Watson, 1986; A-P:-2.6 mm, M-L: +2.0 mm, V:-8.8 mm as measured from bregma). The needle was left in place for 4 min to allow for the 6-OHDA to diffuse into the surrounding region and then slowly withdrawn before cleaning and suturing the wound. Animals were cared for post-operatively as described in section 2.2.4.

Three to four weeks after surgery rats were treated with amphetamine sulphate (2.5 mg/kg i.p. in 0.9 % saline) and rotational behaviour was monitored as described in section 2.3.1. Only rats exhibiting 7 or more ipsilateral turns per minute at peak activity were used for further studies.

3.2.4.2 Establishing AIMs

Rats were primed for dyskinesia, as assessed by AIMs expression (see 3.2.4.4), by daily treatment with L-dopa methyl ester (6.25 mg/kg free base + benserazide 15 mg/kg, dissolved in 0.9 % saline i.p.) for a period of 22 days as described in section 2.3.2.1. Figure 2-4 graphically depicts the development of AIMs in rats over this priming period. All drugs were administered at a volume of 1 ml/kg. After the priming period animals were assessed for AIMs on two subsequent occasions and only rats exhibiting a mean ALO AUC AIMS score >30 were taken forward to the acute challenges.

3.2.4.3 Acute dopaminergic drug challenges in combination with nNOS inhibitors

L-dopa- primed 6-OHDA-lesioned rats were divided into two groups (n=8/group), each balanced for mean AIMs scores based on dyskinesia assessment at the end of the priming period, as described in section 3.2.4.4. In each of the expression studies all drugs were administered according to a modified latin-square design (see Appendix, Figure 0-1) so that each animal received each treatment, and a 3-7 days drug washout period was employed between studies as outlined below (and summarised in **Figure 3-3**).

L-dopa AIMs expression studies

-*Study i.* Animals were treated with L-dopa methyl ester (6.25 mg/kg free base + benserazide 15 mg/kg, in saline 0.9 %, i.p.) plus, at the same time, either ARR17477 (3, 6 or 12 mg/kg in 0.9 % saline s.c.) or saline 0.9 %. AIMs were scored as described in section 3.2.4.4 below. A drug washout period of 1 week was employed between each treatment.

-*Study ii.* Animals were treated with L-dopa methyl ester (6.25 mg/kg + benserazide 15 mg/kg, in saline 0.9 %, i.p.) plus, 30 min earlier, 7-NI (12.5, 25 or 50 mg/kg in DMSO: saline 0.9 %, 50:50, i.p.) or DMSO: saline (0.9 %, 50:50, i.p.). Baseline AIMs were scored as described in section 3.2.4.4, and dopaminergic drugs were administered with scoring continuing from here on. A drug washout period of 3 days was employed between each treatment.

Ropinirole AIMs expression studies

-*Study iii.* Animals were treated with ropinirole (0.2 mg/kg, in saline 0.9 %, i.p.) plus, 30 min beforehand, 7-NI (12.5, 25 or 50 mg/kg in DMSO: saline 0.9 %, 50:50, i.p.) or DMSO: saline (0.9 %, 50:50, i.p.). Baseline AIMs were scored as described in section 3.2.4.4, and dopaminergic drugs were administered with scoring continuing from here on. A drug washout period of 3 days was employed between each treatment.

-*Study iv*. Animals were treated with ropinirole (0.2 mg/kg, in saline 0.9 %, i.p.) plus, at the same time, either ARR17477 (3, 6 or 12 mg/kg in 0.9 % saline s.c.) or saline 0.9 %. AIMs were scored as described in section 3.2.4.4 below. A drug washout period of 1 week was employed between each treatment.

3.2.4.4 AIMs assessment

Dyskinesia was assessed in rats based on the observation of four subtypes of AIM's; locomotive, axial, limb and orolingual (originally described by Cenci et al., 1998), each rated on a scale of 0-4 dependent on frequency and intensity (see section 2.3.2.2). Rats were placed in transparent cages one hour before drug treatment commenced to enable acclimatisation (as described in section 2.3.2.2). Baseline AIMs as described in section 2.3.2.2 were scored 20 min and 5 min prior to dosing. Following drug treatment AIMs were assessed for 5 min every 15 min for up to 210 min.

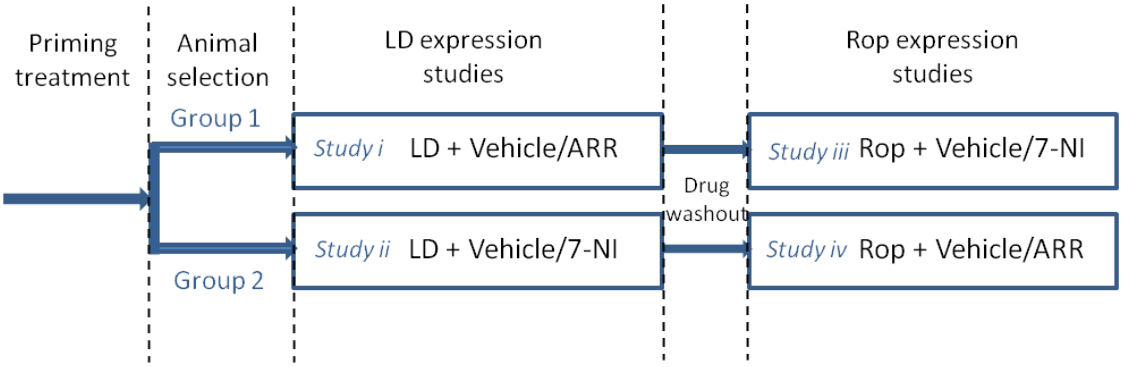

Figure 3-3 Summary diagram of animal groups and treatments used in L-dopa (LD) and ropinirole (Rop) AIMs expression studies in combination with the nNOS inhibitor ARR17477 (ARR) or 7-NI, following priming and selection of 6-OHDA-lesioned rats.

3.2.5 Data and statistical analysis

Time-course data were plotted as the median AIMs score and a mean of the two baseline scores was taken on each assessment day to give a single baseline score plotted at t=0. Data for totals, peaks and duration of activity were plotted as medians and also individual values. Total scores over the assessment period were calculated by AUC (Graphpad Prism version 5.0) using the trapezoid method where each successive 15 min was labelled as a single time-bin and peak score was taken as the maximum AIMs score achieved per 15 min. The duration of AIMs was calculated taking into account all score periods gaining above 0 and in the case of orolingual AIMs included those gaining above 1.

Time-course data were analysed by 2-way-ANOVA followed by Friedman's test and Dunn's post hoc test where appropriate. Total scores, peak scores, and duration of activity were analysed by Friedman's test and Dunn's post hoc test. Statistical significance was set at $p<0.05$ and analyses were carried out in Graphpad Prism 5. Additionally a 'trend' was described in the data where there was greater than a 75 % change from vehicle treated animals.

3.3 Results

3.3.1 Locomotive AIMs in L-dopa-primed 6-OHDA-lesioned rats treated acutely with L-dopa plus nNOS inhibitor

3.3.1.1 nNOS inhibitor + vehicle.

Neither ARR17477 (12 mg/kg) plus vehicle (saline), nor 7-NI (50 mg/kg) plus vehicle (saline) produced locomotive AIMs in 6-OHDA-lesioned rats primed with L-dopa (**Figure 3-4a & Figure 3-5a**).

3.3.1.2 Vehicle + L-dopa.

L-dopa alone induced locomotive AIMs in Group 1 and 2 for between 15-135 min (**Figure 3-4a & Figure 3-5a**) with a peak score of 2 (**Figure 3-4c & Figure 3-5c**), and a duration of locomotive AIMs activity of between 120-135 min (**Figure 3-4d & Figure 3-5d**). The total AIMs score for the session was 12-14 (**Figure 3-4b & Figure 3-5b**).

3.3.1.3 nNOS inhibitor + L-dopa.

There was no significant effect of ARR17477 (3, 6 or 12 mg/kg) or 7-NI (12.5, 25, or 50 mg/kg) on L-dopa-induced locomotive AIMs in either group as measured by peak (**Figure 3-4c & Figure 3-5c**), duration of activity (**Figure 3-4d & Figure 3-5d**) or total AIMs score (**Figure 3-4b & Figure 3-5b**).

3.3.2 Locomotive AIMs in L-dopa-primed 6-OHDA-lesioned rats treated acutely with ropinirole plus nNOS inhibitor

3.3.2.1 nNOS inhibitor + vehicle

As previously described neither ARR17477 (12 mg/kg) plus vehicle (saline), or 7-NI (50 mg/kg) plus vehicle (saline) produced locomotive AIMs in L-dopa-primed 6-OHDA-lesioned rats (**Figure 3-6a & Figure 3-7a**).

3.3.2.2 Vehicle + ropinirole

Ropinirole alone induced locomotive AIMs from between 15-60 min (**Figure 3-6a & Figure 3-7a**) with a peak score of 1.5-2.5 (**Figure 3-6c & Figure 3-7c**) and duration of activity of between 20-60 min (**Figure 3-6d & Figure 3-7d**). Ropinirole produced locomotive AIMs of a similar severity level to L-dopa, but these lasted for a shorter time period. The total AIMs score was 2-5 (**Figure 3-6b & Figure 3-7b**).

3.3.2.3 nNOS inhibitor + ropinirole

No significant effect of ARR17477 (3, 6 or 12 mg/kg) was observed on ropinirole induced locomotive AIMs as measured by peak scores (**Figure 3-6c**), duration of activity (**Figure 3-6d**), or totals (**Figure 3-6b**). Surprisingly 7-NI caused a significant increase in the duration of ropinirole-induced locomotive AIMs at specific time points (**Figure 3-7a**). AIMs scores were significantly greater than for ropinirole alone at 45

min (7-NI 12.5 mg/kg), at 60 min (7-NI 25 mg/kg) and at 60-90 min (7-NI 50 mg/kg), translating into an extension in the duration of ropinirole-induced AIMs. No overall significant effect of 7-NI (12.5, 25, or 50 mg/kg) was observed on peak AIMs (**Figure 3-7c**). However, the duration of locomotive AIMs was significantly extended by 7-NI 12.5 mg/kg, although the change did not reach statistical significance with higher doses of the nNOS inhibitor (**Figure 3-7d**). Meanwhile this pattern was not reflected in the total AIMs score which was not significantly affected by 7-NI at any dose, although total AIMs did tend to be increased by 7-NI at all doses (**Figure 3-7b**).

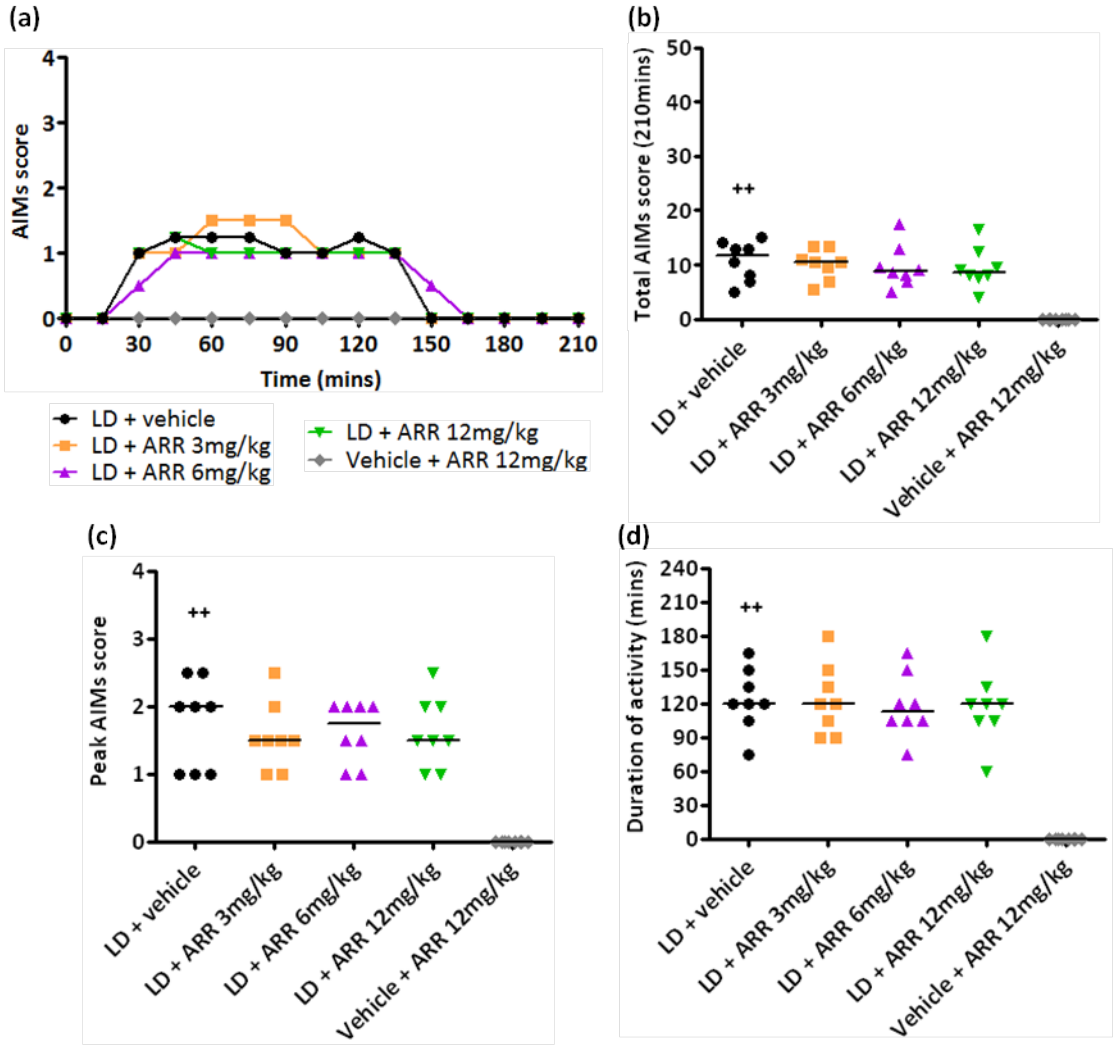

Figure 3-4 Locomotive AIMs expression following ARR17477 plus L-dopa treatment. ARR17477 (ARR; 3, 6 or 12 mg/kg s.c) and L-dopa (LD; 6.25 mg/kg plus benserazide 15 mg/kg i.p.) treatment in L-dopa-primed 6-OHDA-lesioned rats. Data are presented as medians (n=8); **(a)** Time-course, and also individual values **(b)** Total, **(c)** Peak and **(d)** Duration of activity. ++p<0.01 compared to vehicle + ARR 12 mg/kg s.c. treatment. Time-course data were analysed by 2-way-ANOVA and Friedman's test followed by Dunn's post hoc test, and all other data were analysed by Friedman's test followed by Dunn's post hoc test.

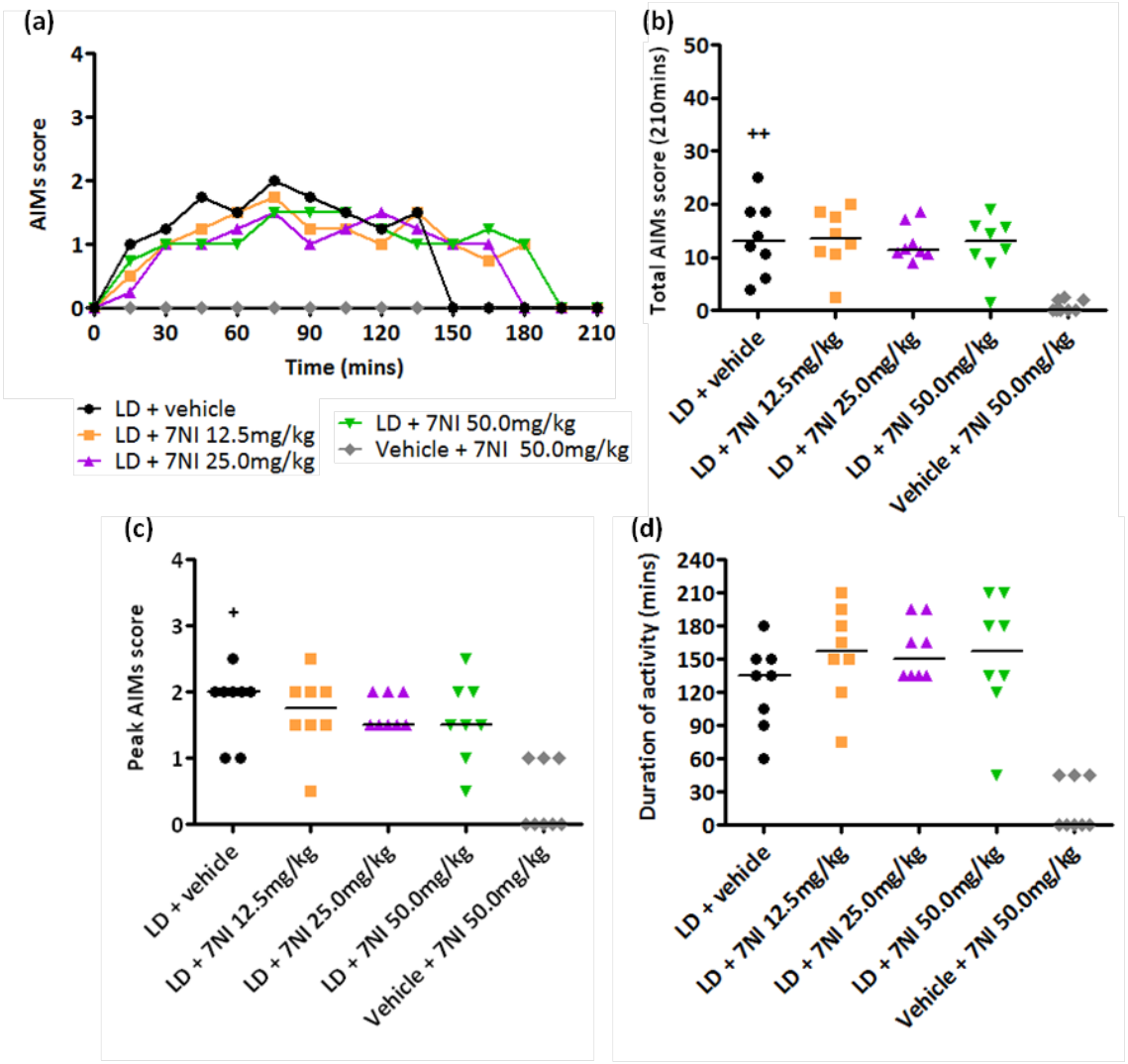

Figure 3-5 Locomotive AIMs expression following 7-NI plus L-dopa treatment. 7-NI (12.5, 25.0 or 50.0 mg/kg i.p) and L-dopa (LD; 6.25 mg/kg plus benserazide 15 mg/kg i.p.) treatment in L-dopa-primed 6-OHDA-lesioned rats. Data are presented as medians (n=8); **(a)** Time-course, and also individual values **(b)** Total, **(c)** Peak and **(d)** Duration of activity. +p<0.05, ++p<0.01 compared to vehicle + 7-NI 50 mg/kg i.p. treatment. Time-course data were analysed by 2-way-ANOVA and Friedman's test followed by Dunn's post hoc test, and all other data were analysed by Friedman's test followed by Dunn's post hoc test.

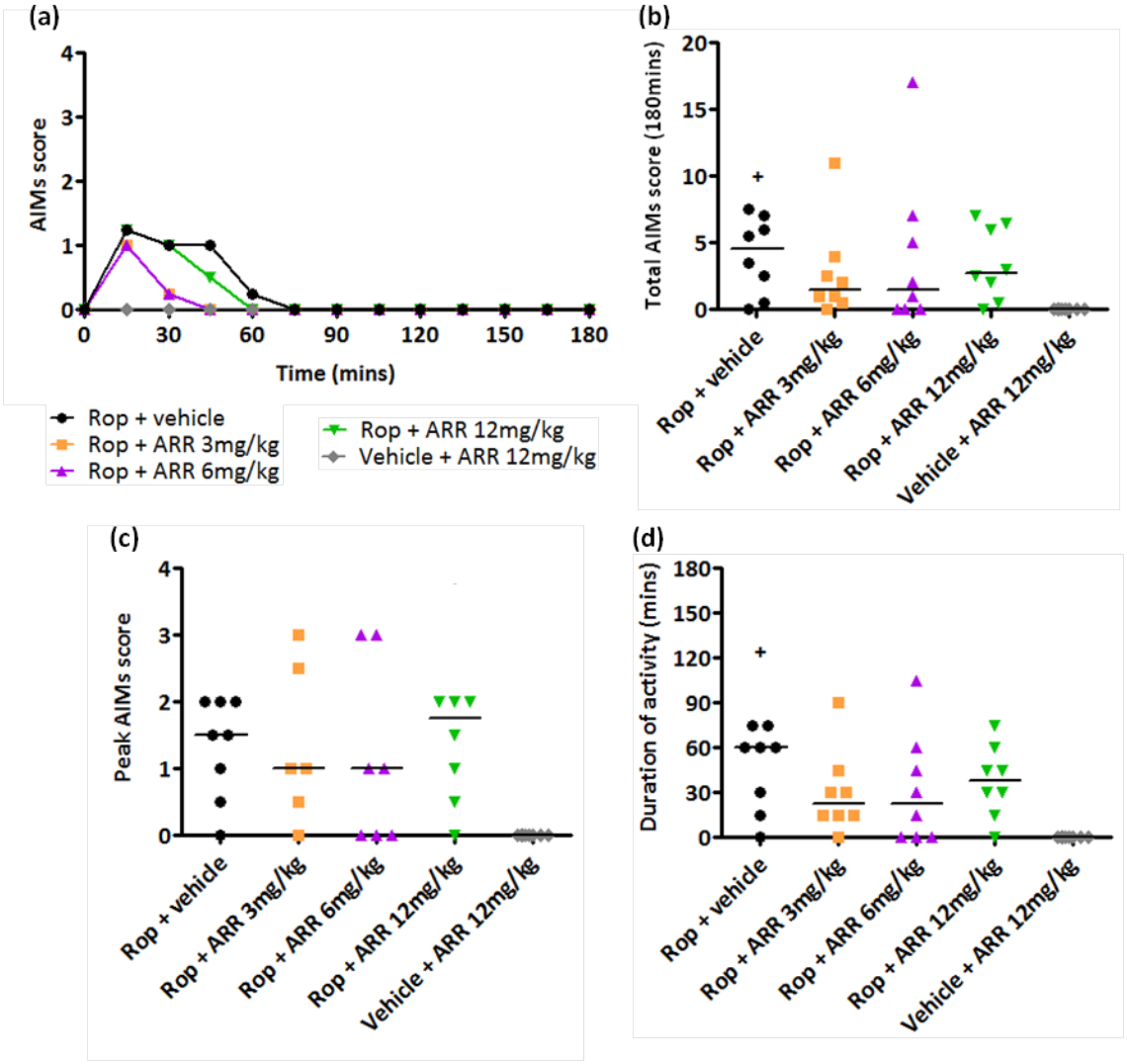

Figure 3-6 Locomotive AIMs expression following ARR17477 plus ropinirole treatment. ARR17477 (ARR; 3, 6 or 12 mg/kg s.c) and ropinirole (Rop; 0.2 mg/kg s.c.) treatment in L-dopa-primed 6-OHDA-lesioned rats. Data are presented as medians (n=8); **(a)** Time-course, and also individual values **(b)** Total, **(c)** Peak and **(d)** Duration of activity. +p<0.05 compared to vehicle + ARR 12 mg/kg s.c. treatment. Time-course data were analysed by 2-way-ANOVA and Friedman's test followed by Dunn's post hoc test, and all other data were analysed by Friedman's test followed by Dunn's post hoc test.

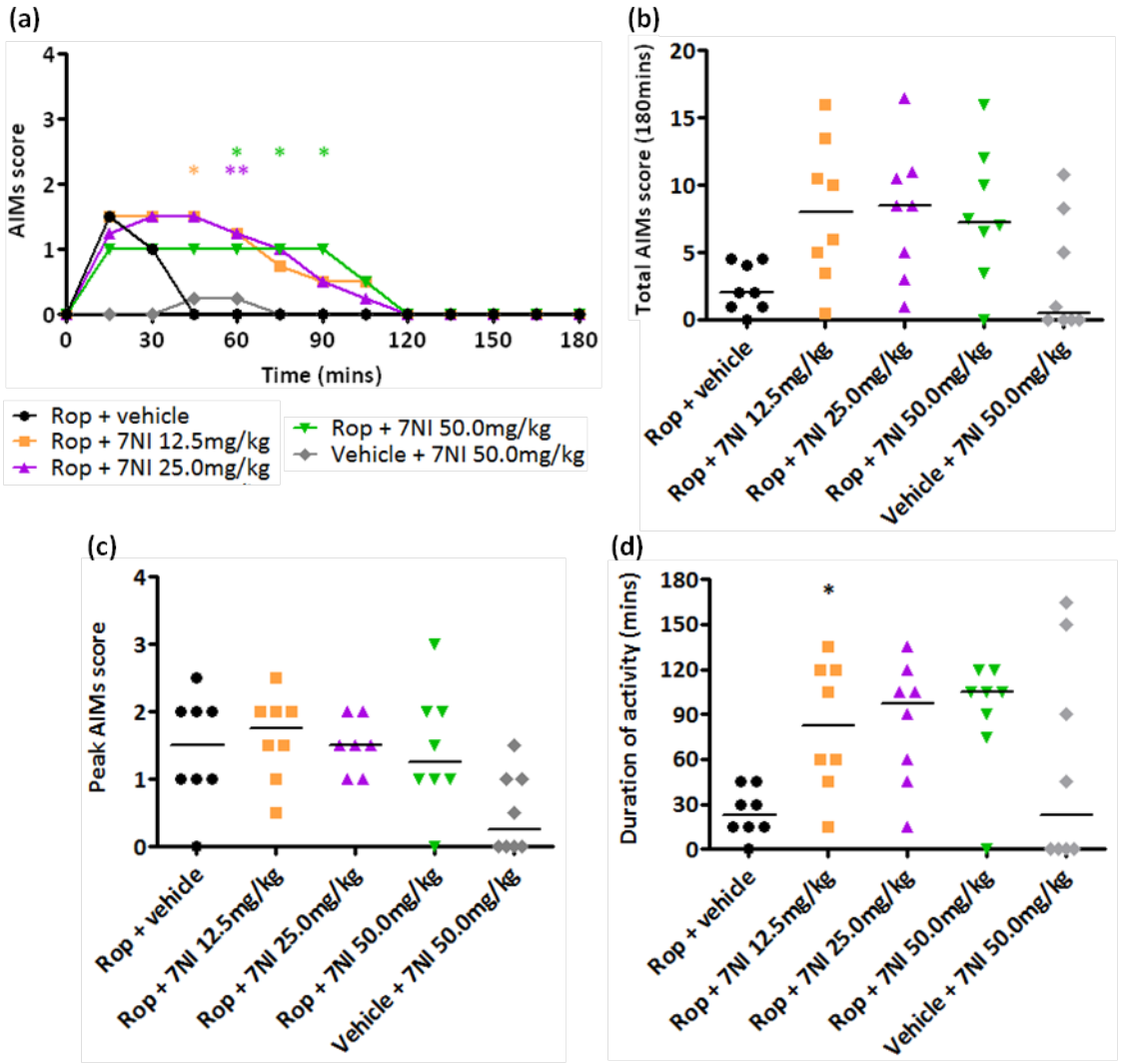

Figure 3-7 Locomotive AIMs expression following 7-NI treatment plus ropinirole. 7-NI (12.5, 25.0 or 50.0 mg/kg mg/kg i.p.) and ropinirole (Rop; 0.2 mg/kg s.c.) treatment in L-dopa-primed 6-OHDA-lesioned rats. Data are presented as medians (n=8); **(a)** Time-course, and also individual values **(b)** Total, **(c)** Peak and **(d)** Duration of activity. *p<0.05, **p<0.01 compared to rop + vehicle treatment (colours used in (a) refer to key). Time-course data were analysed by 2-way-ANOVA and Friedman's test followed by Dunn's post hoc test, and all other data were analysed by Friedman's test followed by Dunn's post hoc test.

3.3.3 Axial, limb, orolingual and ALO AIMs in L-dopa-primed 6-OHDA-lesioned rats treated acutely with L-dopa plus nNOS inhibitor

3.3.3.1 nNOS inhibitor + vehicle

Rats treated with ARR17477 (12mg/kg) plus vehicle (saline), or 7-NI (50 mg/kg) plus vehicle (saline) did not produce any axial or limb AIMs, and exhibited little or no orolingual and ALO AIMs (**Figure 3-8a-d & Figure 3-10a-d**).

3.3.3.2 Vehicle + L-dopa

L-dopa alone induced axial AIMs from 15-165 min predominantly of a moderate to marked nature (**Figure 3-8a & Figure 3-10a**), with a peak score of 3-3.5 across both groups (**Figure 3-9a & Figure 3-11a**). Both groups also showed axial AIMs for an average duration of 160 min (**Figure 3-9a' & Figure 3-11a'**) with a total AIMs score of 23-27 (**Figure 3-8a' & Figure 3-10a'**).

Limb AIMs were induced over the same time frame as axial AIMs (**Figure 3-8b & Figure 3-10b**), were mainly of moderate intensity peaking at a score of 2.5-2.75 (**Figure 3-9b & Figure 3-11b**) with a duration of activity of 160-165 min (**Figure 3-9b' & Figure 3-11b').** Limb AIMs totalled 22-24 (**Figure 3-8b' & Figure 3-10b'**).

Meanwhile orolingual AIMs of mild to marked intensity were predominantly induced between 15-180 min and observed fleetingly at later time points (**Figure 3-8c & Figure 3-10c**), with an overall peak score of 3-4 **(Figure 3-9c & Figure 3-11c)** and duration of activity of 130-165 min (**Figure 3-9c' & Figure 3-11c').** Orolingual AIMs totalled 20-28 (**Figure 3-8c' & Figure 3-10c'**).

ALO AIMs scores reflected individual AIMs categories for L-dopa alone with animals expressing moderate to marked ALO AIMs from 15-165 min (**Figure 3-8d & Figure 3-10d**). Both groups showed a peak ALO AIMs score of 8-8.5 (**Figure 3-9d & Figure 3-11d**) and marked AIMs were maintained for a period of at least 90 min before gradually declining to baseline levels by 180-195 min (**Figure 3-8d & Figure 3-10d**). ALO AIMs were expressed for an overall duration of 165 min (**Figure 3-9d' & Figure 3-11d'**) with a total AIMs score of 70-77 (**Figure 3-8d' & Figure 3-10d'**).

3.3.3.3 nNOS inhibitor + L-dopa

There was no significant effect of ARR17477 (3, 6 or 12mg/kg) on axial, limb, orolingual or ALO AIMs induced by L-dopa as measured by peak score (**Figure 3-9a-d**), duration of activity (**Figure 3-9a'-d'**) or total AIMs (**Figure 3-8a'-d'**).

By contrast 7-NI (50 mg/kg) significantly increased the duration of L-dopa-induced axial AIMs as measured at individual time points without increasing severity (**Figure 3-10a**). Axial AIMs remained raised at moderate-marked intensity levels at 165 and 180 min following L-dopa plus 7-NI (50 mg/kg) treatment, compared to L-dopa alone which resulted in axial AIMs that were mild or absent by these respective time points (**Figure 3-10a**). However there was no statistically significant effect of 7-NI (50

mg/kg or 12.5 and 25 mg/kg) on overall axial AIMs as measured by duration (**Figure 3-11a'**), peak (**Figure 3-11a**) or total AIMs score (**Figure 3-10a'**).

7-NI (25 and 50 mg/kg) significantly increased the duration of L-dopa-induced limb AIMs at 180 min with moderate levels still observable compared to L-dopa only treatment for which limb AIMs were absent at this same time point (**Figure 3-10b**). However there was no overall effect of 7-NI treatment on peak score (**Figure 3-11b**), duration (**Figure 3-11b'**) or total limb AIMs (**Figure 3-10b'**).

7-NI (12.5 mg/kg) significantly extended the duration of moderate orolingual AIMs at 180 min and this was also the case for 7-NI (50 mg/kg) at 195 min compared to L-dopa alone (**Figure 3-10c**). Again, there was no overall effect of 7-NI treatment on orolingual peak score (**Figure 3-11c**), duration (**Figure 3-11c'**) or total AIMs (**Figure 3-10c'**).

7-NI (12.5 mg/kg) significantly extended the duration of moderate ALO AIMs at 180 and 195 min, and this was also true for 7-NI 50 mg/kg at 180-210 min, compared to L-dopa alone (**Figure 3-10d**). 7-NI (50 mg/kg) also caused a significant increase in the overall duration of activity of ALO AIMs by 45 min compared to treatment with L-dopa alone (**Figure 3-11d'**). However there was no significant effect of 7-NI (12.5, 25, or 50 mg/kg) on L-dopa induced ALO AIMs as measured by peak scores or total AIMs (**Figure 3-11d & Figure 3-10d'**).

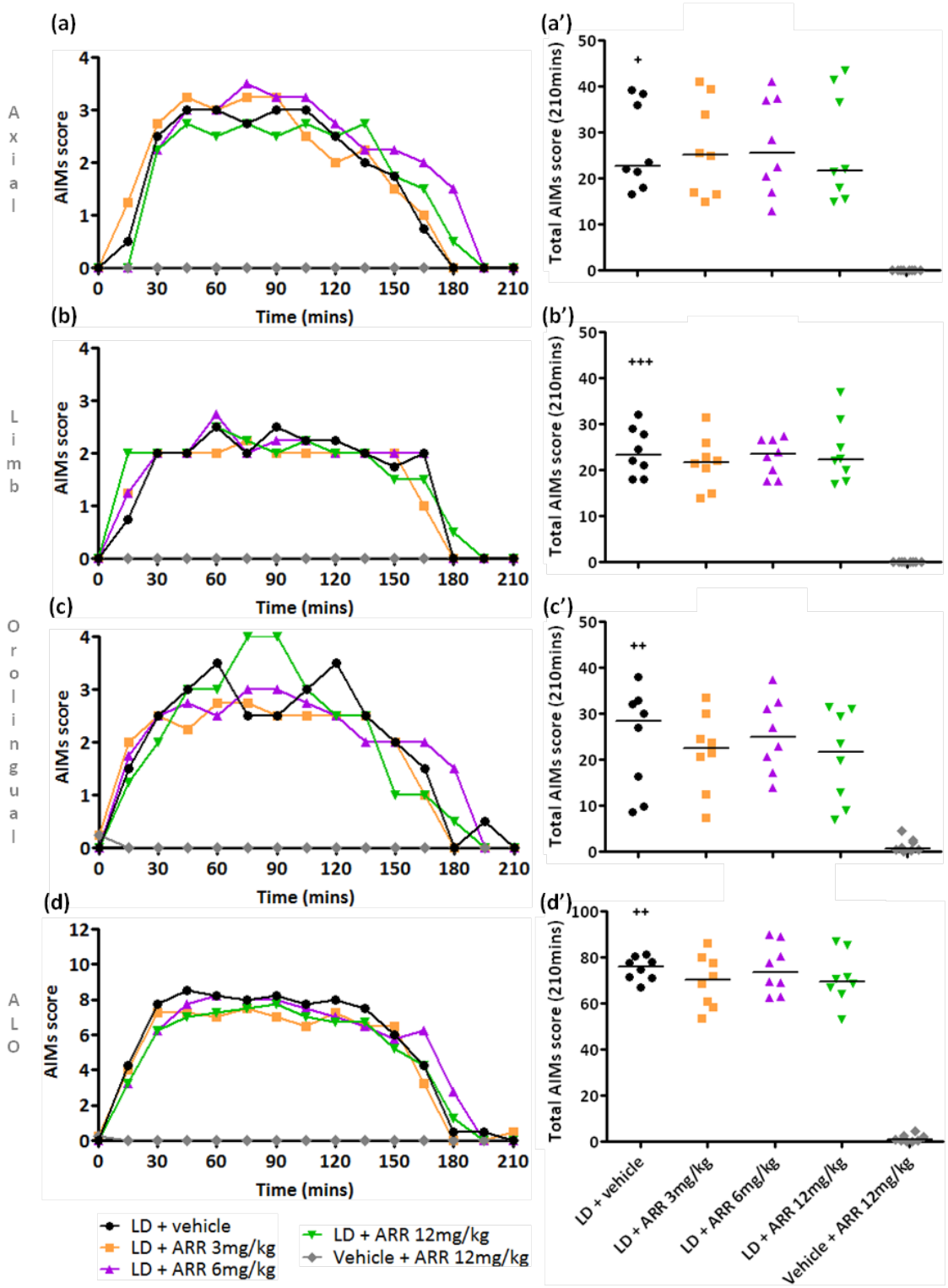

Figure 3-8 Time-course and Total score for axial (a & a'), limb (b & b'), orolingual (c & c') and ALO AIMs (d & d') expression following ARR17477 plus L-dopa treatment. ARR17477 (ARR); 3, 6 or 12 mg/kg s.c) and L-dopa (LD; 6.25 mg/kg plus benserazide 15 mg/kg i.p.) treatment in L-dopa-primed 6-OHDA-lesioned rats. Data are presented as medians (n=8); **(a-d)** Time-course, and also individual values **(a'-d')** Total; +p<0.05, ++p<0.01, +++ p<0.001 compared to vehicle + ARR 12 mg/kg s.c. treatment. Time-course data were analysed by 2-way-ANOVA and Friedman's test followed by Dunn's post hoc test, and all other data were analysed by Friedman's test followed by Dunn's post hoc test.

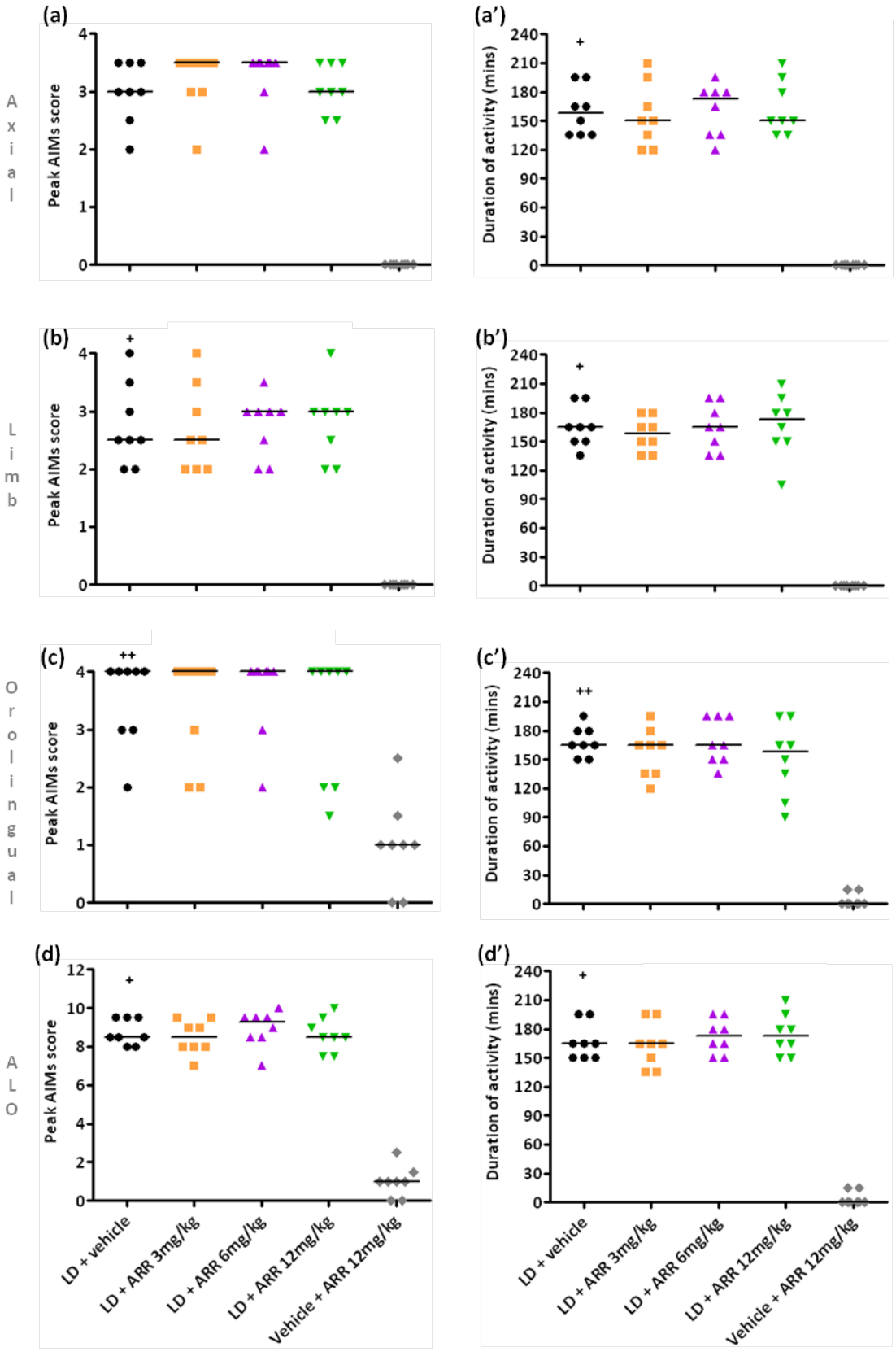

Figure 3-9 Peak scores and Duration of activity for axial (a & a'), limb (b & b'), orolingual (c & c') and ALO AIMs (d & d') expression following ARR17477 plus L-dopa treatment. ARR17477 (ARR; 3, 6 or 12 mg/kg s.c) and L-dopa (LD; 6.25 mg/kg plus benserazide 15 mg/kg i.p.) treatment in L-dopa-primed 6-OHDA-lesioned rats. Data are presented as medians and individual values (n=8); **(a-d)** Peak score and **(a'-d')** Duration of activity; +p<0.05, ++p<0.01 compared to vehicle + ARR 12 mg/kg s.c. treatment. Data were analysed by Friedman's test followed by Dunn's post hoc test.

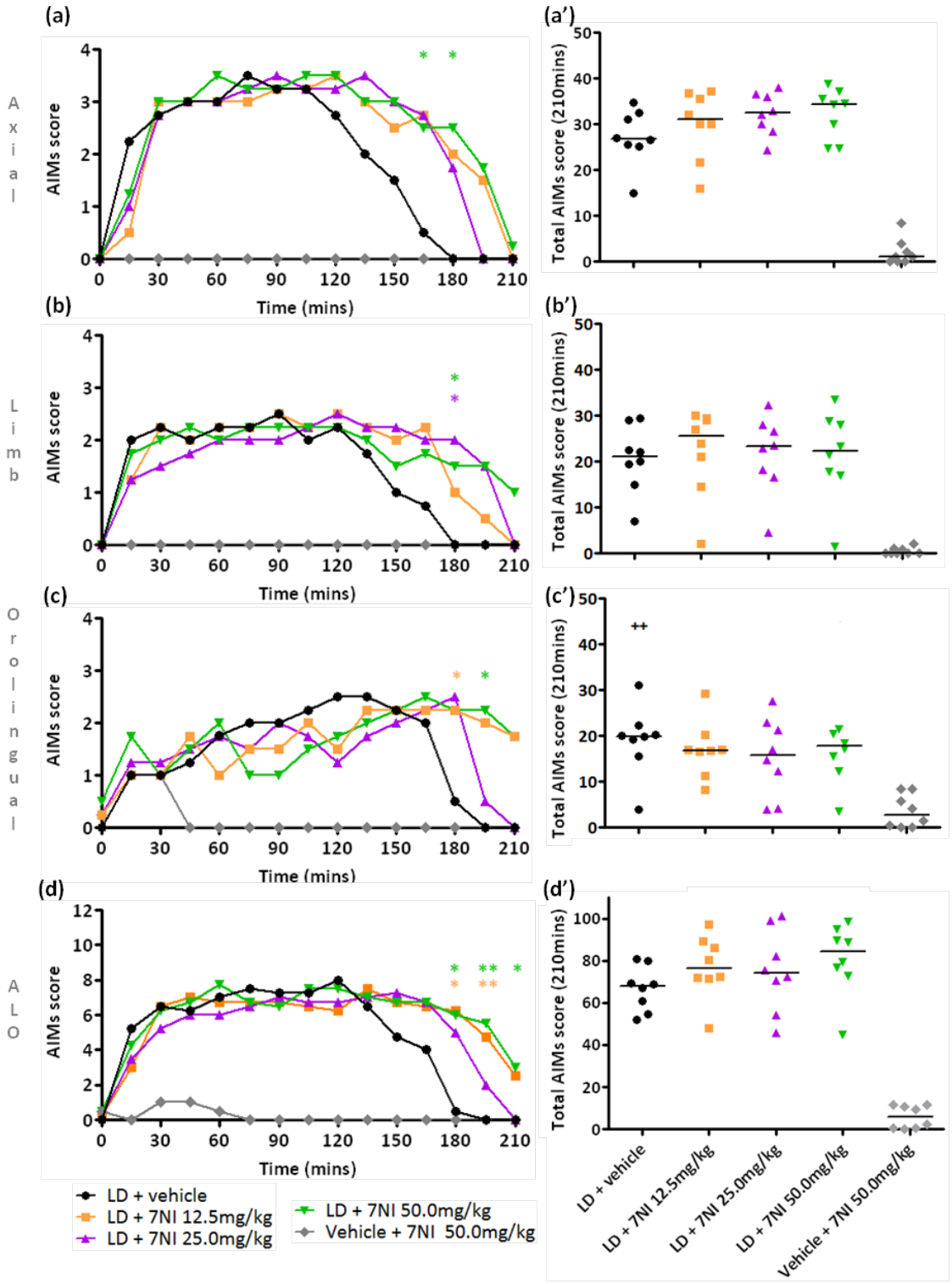

Figure 3-10 Time-course and Total score for axial (a & a'), limb (b & b'), orolingual (c & c') and ALO AIMs (d & d') expression following 7-NI plus L-dopa treatment. 7-NI (12.5, 25.0 or 50.0 mg/kg i.p.) and L-dopa (LD; 6.25 mg/kg plus benserazide 15 mg/kg i.p.) treatment in L-dopa-primed 6-OHDA-lesioned rats. Data are presented as medians (n=8); **(a-d)** Time-course, and also individual values **(a'-d')** Total; *p<0.05, **p<0.01 compared to LD + vehicle treatment (colours used in (a-d) refer to key), ++p<0.01 compared to vehicle + 7-NI 50 mg/kg i.p. treatment. Time-course data were analysed by 2-way-ANOVA and Friedman's test followed by Dunn's post hoc test, and all other data were analysed by Friedman's test followed by Dunn's post hoc test.

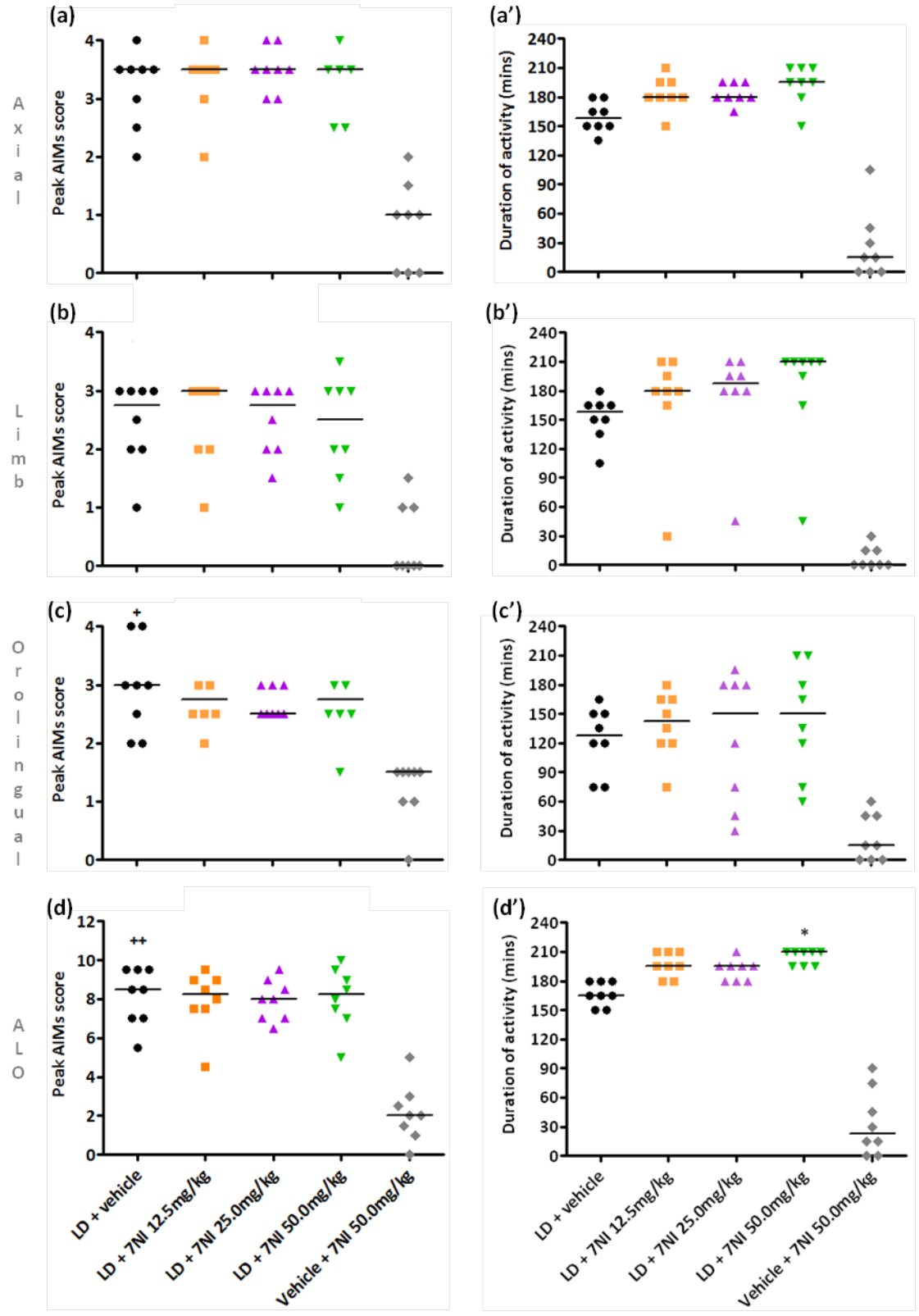

Figure 3-11 Peak scores and Duration of activity for axial (a & a'), limb (b & b'), orolingual (c & c') and ALO AIMs (d & d') expression following 7-NI plus L-dopa treatment. 7-NI (12.5, 25.0 or 50.0 mg/kg i.p.) and L-dopa (LD; 6.25 mg/kg mg/kg plus benserazide 15 mg/kg i.p.) treatment in L-dopa-primed 6-OHDA-lesioned rats. Data are presented as medians and individual values (n=8); **(a-d)** Peak score and **(a'-d')** Duration of activity; *p<0.05 compared to LD + vehicle treatment, +p<0.05, ++p<0.01 compared to vehicle + 7-NI 50 mg/kg i.p. treatment. Data were analysed by Friedman's test followed by Dunn's post hoc test.

3.3.4 Axial, limb, orolingual and ALO AIMs in L-dopa-primed 6-OHDA-lesioned rats treated acutely with ropinirole plus nNOS inhibitor

3.3.4.1 nNOS inhibitor + vehicle

Animals treated with ARR17477 (12mg/kg) plus vehicle (saline) did not exhibit any axial, limb, or orolingual AIMs and consequently no ALO AIMs were evident (**Figure 3-12a-d**). By contrast animals treated with 7-NI (50 mg/kg) and vehicle (saline) showed occasional mild and fleeting axial AIMs between 30-135 min (**Figure 3-14a**) with a peak score of 1 (**Figure 3-15a**) and median duration of 30 min (**Figure 3-15a'**) and were observed in 7 out of 8 of the animals. However limb AIMs were not observed (**Figure 3-14b**) and mild orolingual AIMs appeared only briefly (**Figure 3-14c**) with a peak score of 1 (**Figure 3-15c**). Mild and fleeting ALO AIMs were therefore observed between 15 and 135 min and did not exceed a score of 1 at any individual time point (**Figure 3-14d**). It is noteworthy that comparison of the total AIMs scores for this group of animals treated with 7-NI (50 mg/kg) plus vehicle and the other group used for the L-dopa plus nNOS inhibitor study receiving the same treatment (see **Figure 3-14a' & d', & Figure 3-10a' & d'**) shows that there is no significant difference overall in axial or ALO AIMS (Mann Whitney test; data not shown).

3.3.4.2 Vehicle + ropinirole

Ropinirole alone induced axial AIMs from 15-75 min (**Figure 3-12a & Figure 3-14a**) with a peak score at 15 min of 2.5-3 (**Figure 3-13a & Figure 3-15a**) and declining thereafter. The total duration of axial AIMs was between 60-70 min and these were moderate to marked in severity (**Figure 3-13a' & Figure 3-15a'**), with a total AIM's score of 8-12 (**Figure 3-12a' & Figure 3-14a'**).

Moderate to marked limb AIMs were induced in animals from 15 min onwards declining to baseline levels by between 45 and 75 min (**Figure 3-12b & Figure 3-14b**), showing a peak score of 2-3 (**Figure 3-13b & Figure 3-15b**) and duration of activity ranging from 25-60 min (**Figure 3-13b' & Figure 3-15b'**). Overall limb AIMs totalled 4-10 (**Figure 3-12b' & Figure 3-14b'**).

Mild and intermittent orolingual AIMs were induced between 15 and 120 min (**Figure 3-12c & Figure 3-14c**) after ropinirole treatment, peaking at a score of 1.5-2 (**Figure 3-13c & Figure 3-15c**) and lasting for a median duration of between 25-30 min (**Figure 3-13c' & Figure 3-15c'**). Overall orolingual AIMs totalled 4-5 (**Figure 3-12c' &Figure 3-14c'**).

Ropinirole treated rats exhibited moderate to marked ALO AIMs between 15 and 90 min (**Figure 3-12d & Figure 3-14d**). Scores peaked at 4.5-6.5 (**Figure 3-13d & Figure 3-15d**), and gradually returned to baseline levels by 105 min with a minor fluctuation after this time. ALO AIMS were exhibited for an overall duration of 60-75 min (**Figure 3-13d' & Figure 3-15d'**) and totalled 15-26 (**Figure 3-13d' & Figure 3-15d'**).

3.3.4.3 nNOS inhibitor + ropinirole

Overall there was no significant effect of ARR17477 (3, 6 or 12mg/kg) on peak score (**Figure 3-13a-d**), duration of activity (**Figure 3-13a'- d'**) or total AIMs (**Figure 3-12a'-d'**), for axial, limb, orolingual or ALO AIMs.

By contrast, 7-NI (50 mg/kg) produced a significant increase in duration of ropinirole-induced axial AIMs of moderate-marked levels at individual assessment periods between 75-105 min compared to ropinirole treatment alone (**Figure 3-14a**). 7-NI (50 mg/kg) also caused a significant increase in axial overall duration of activity by 75 min (**Figure 3-15a'**) and total AIMs of 11 (**Figure 3-14a'**). There was no significant effect of 7-NI (12.5-50 mg/kg) on peak axial AIMs score (**Figure 3-15a**), hence 7-NI had no effect on severity of axial AIMs, or of 7-NI at the lower two doses (12.5 and 25 mg/kg) on overall duration (**Figure 3-15a'**) or total axial AIMs (**Figure 3-14a'**).

7-NI (25 and 50 mg/kg) caused a significant increase in duration of moderate limb AIMs at 45 min, 7-NI (25 mg/kg) at 60min and 7-NI (50 mg/kg) at 75 and 90 min (**Figure 3-14b**) compared to ropinirole treatment alone. 7-NI (50 mg/kg) also caused a significant increase in total limb AIMs of 10 (**Figure 3-14**). There was no significant effect of 7-NI (12.5-50 mg/kg) on peak limb AIMs (**Figure 3-15b**), or overall duration of limb AIMs (**Figure 3-15b'**) and also no effect of 7-NI (12.5 and 25 mg/kg) on total limb AIMs (**Figure 3-14b'**) as compared to ropinirole alone.

Orolingual AIMs were unaffected by 7-NI treatment at individual time points (**Figure 3-14c**) and also in terms of overall peak, duration and total AIMs (**Figure 3-14c', Figure 3-15 c & c'**).

7-NI (25 mg/kg) caused a significant increase in the duration of moderate ALO AIMs at individual assessment periods of 45 and 75 min compared to treatment with ropinirole alone (**Figure 3-14d**). This increased duration was similarly the case for 7-NI (50 mg/kg) at 75-105 min and also at 135 min (**Figure 3-14d**). Treatment with the highest dose of 7-NI (50 mg/kg) plus ropinirole caused a significant increase in ALO AIMs overall as measured by duration of activity (**Figure 3-15d'**) and total AIMs (**Figure 3-14d'**). The duration of activity of ALO AIMs following 7-NI (50 mg/kg) was extended by 75 min as compared to treatment with ropinirole alone (**Figure 3-15d'**) and total AIMs were increased by a score of 20 (**Figure 3-14d'**). No significant effects of lower doses of 7NI (12.5 and 25 mg/kg) were observed on ALO AIMs as measured by peak, duration or total AIMs (**Figure 3-15d & d', & Figure 3-14d'**).

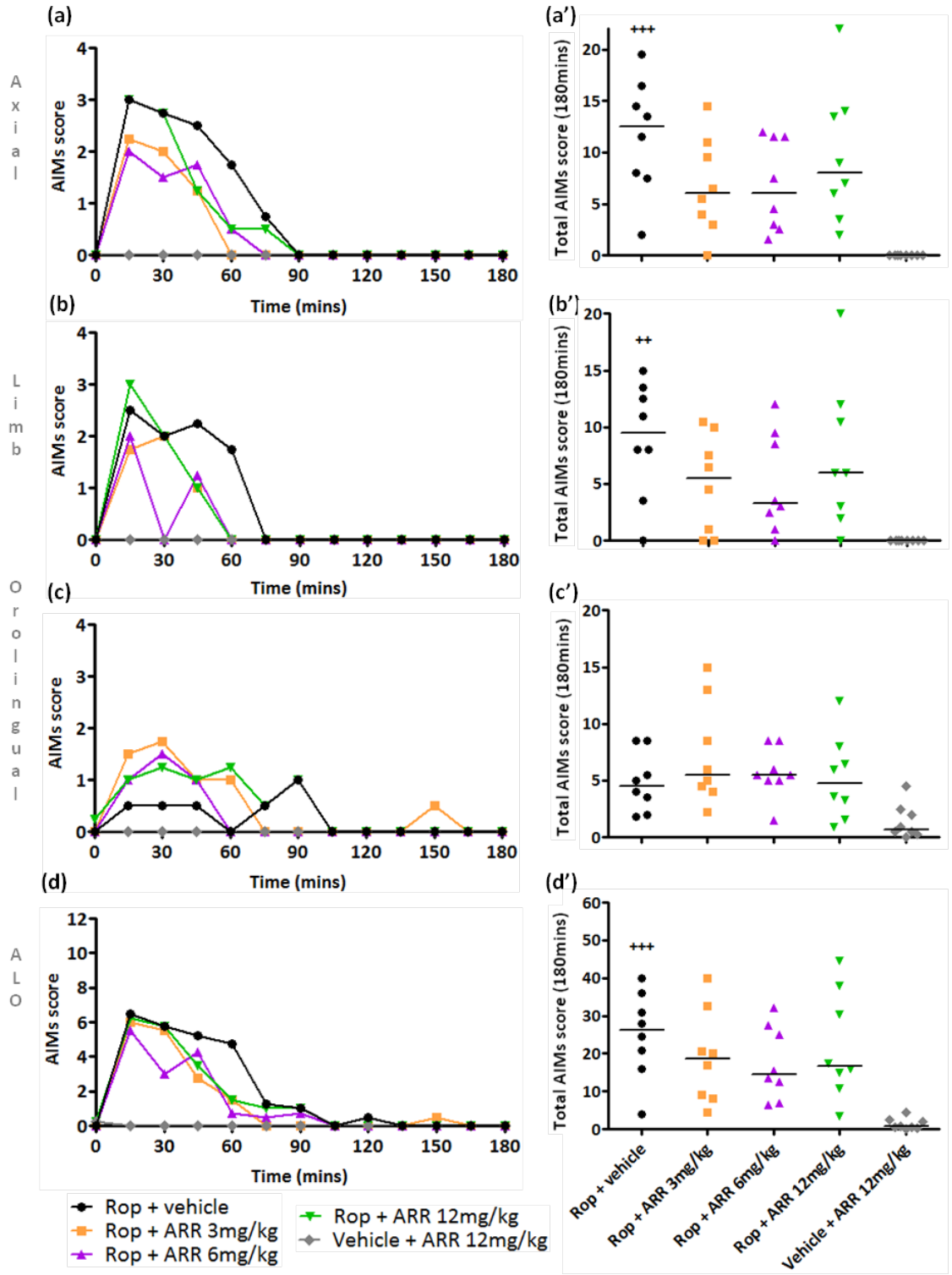

Figure 3-12 Time-course and Total score for axial (a & a'), limb (b & b'), orolingual (c & c') and ALO AIMs (d & d') expression following ARR17477 plus ropinirole treatment. ARR17477 (ARR; 3, 6 or 12 mg/kg s.c) and ropinirole (Rop; 0.2 mg/kg i.p.) treatment in L-dopa-primed 6-OHDA-lesioned rats. Data are presented as medians (n=8); **(a-d)** Time-course, and also individual values **(a'-d')** Total; ++p<0.01, +++p<0.001 compared to vehicle + ARR 12 mg/kg s.c. Time-course data were analysed by 2-way-ANOVA and Friedman's test followed by Dunn's post hoc test, and all other data were analysed by Friedman's test followed by Dunn's post hoc test.

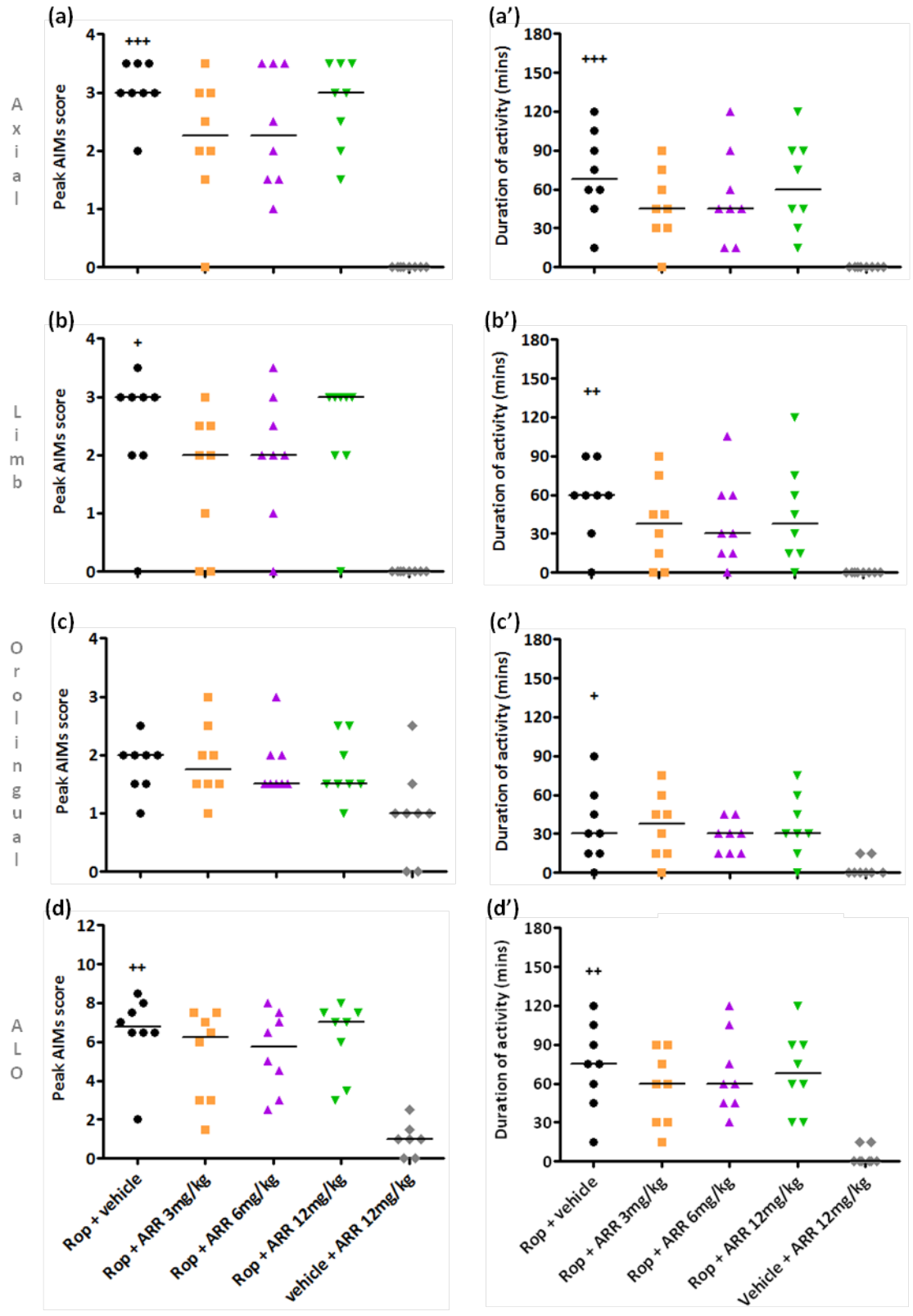

Figure 3-13 Peak scores and Duration of activity for axial (a & a'), limb (b & b'), orolingual (c & c') and ALO AIMs (d & d') expression following ARR17477 plus ropinirole treatment. ARR17477 (ARR; 3, 6 or 12 mg/kg s.c) and ropinirole (Rop; 0.2 mg/kg i.p.) treatment in L-dopa-primed 6-OHDA-lesioned rats. Data are presented as medians and individual values (n=8); **(a-d)** Peak score and **(a'-d')** Duration of activity;. +p<0.05, ++p<0.01, +++ p<0.001 compared to vehicle + ARR 12 mg/kg s.c treatment. Data were analysed by Friedman's test followed by Dunn's post hoc test.

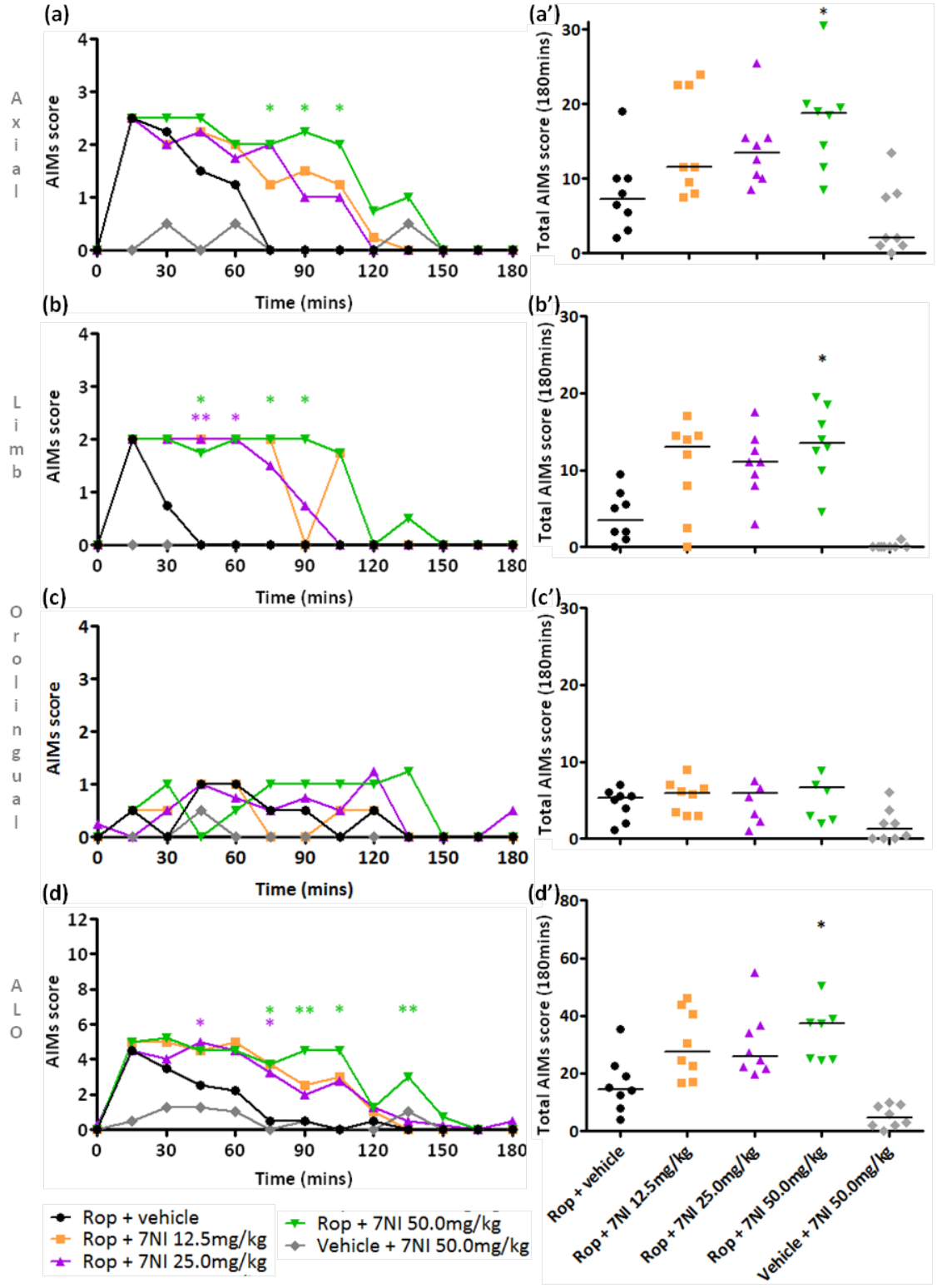

Figure 3-14 Time-course and Total score for axial (a & a'), limb (b & b'), orolingual (c & c') and ALO AIMs (d & d') expression following 7-NI plus ropinirole treatment. 7-NI (12.5, 25.0 or 50.0 mg/kg i.p.) and ropinirole (Rop; 0.2 mg/kg i.p.) treatment in L-dopa-primed 6-OHDA-lesioned rats. Data are presented as medians (n=8); **(a-d)** Time-course, and also individual values **(a'-d')** Total; *p<0.05, **p<0.01 compared to rop + vehicle treatment (colours used in (a-d) refer to key). Time-course data were analysed by 2-way-ANOVA and Friedman's test followed by Dunn's post hoc test, and all other data were analysed by Friedman's test followed by Dunn's post hoc test.

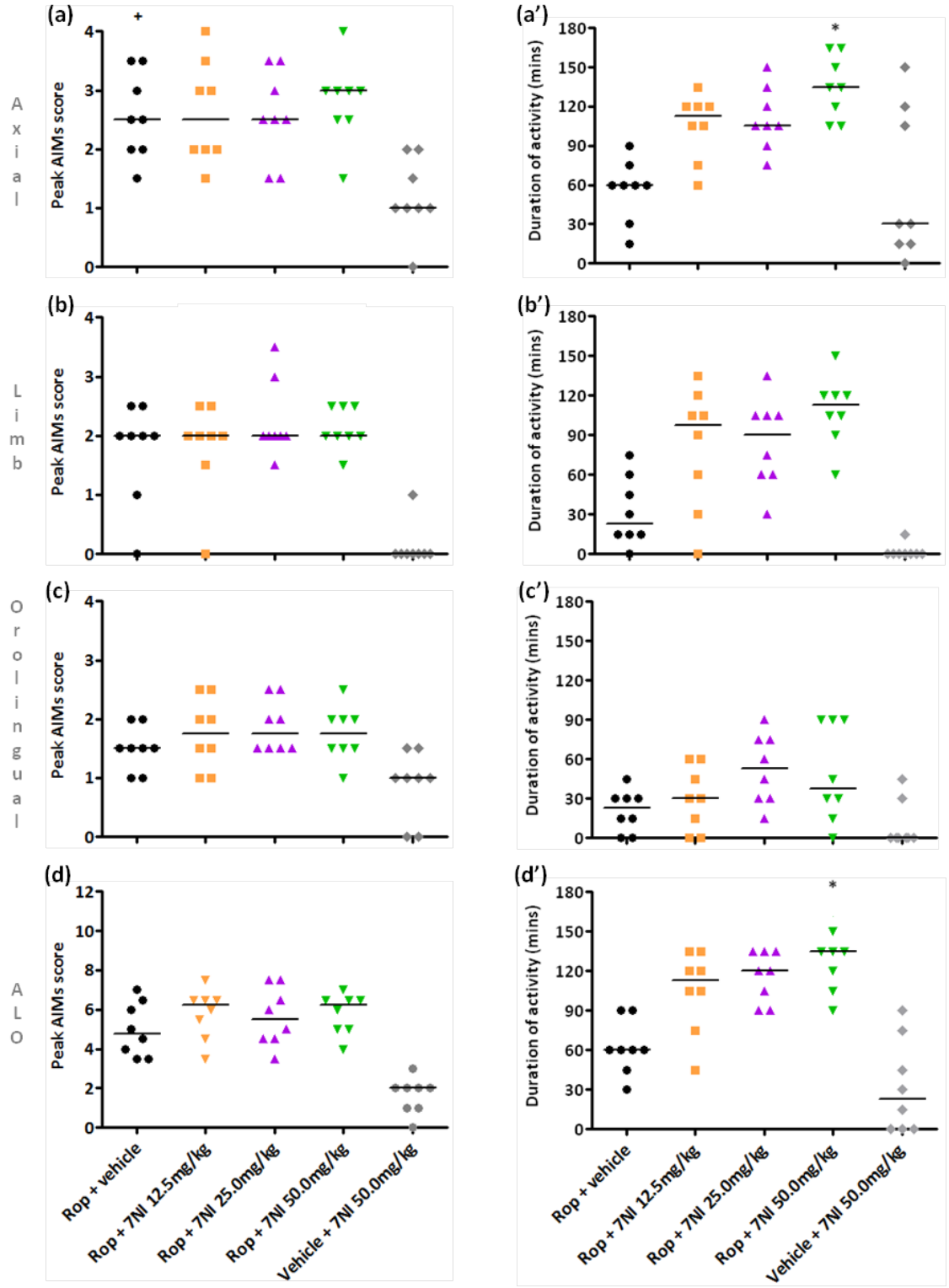

Figure 3-15 Peak scores and Duration of activity for axial (a & a'), limb (b & b'), orolingual (c & c') and ALO AIMs (d & d') expression following 7-NI plus ropinirole treatment. 7-NI (12.5, 25.0 or 50.0 mg/kg i.p.) and ropinirole (Rop; 0.2 mg/kg i.p.) treatment in L-dopa-primed 6-OHDA-lesioned rats. Data are presented as medians and individual values (n=8); **(a-d)** Peak score and **(a'-d')** Duration of activity; *p<0.05 compared to rop + vehicle treatment. +p<0.05 compared to vehicle + 7-NI treatment. Data were analysed by Friedman's test followed by Dunn's post hoc test.

3.4 Discussion

The studies described in this chapter set out to establish how nNOS activity in the brain is affected by two inhibitors ARR17477 and 7-NI described in the literature, and subsequently to evaluate their capacity to treat established dyskinesia in PD. It was hypothesised that the selective inhibition of neuronal NOS would reduce the expression of dyskinesia by L-dopa or the dopamine agonist ropinirole, and this was tested in the 6-OHDA-lesioned rat primed to express dyskinesia by chronic L-dopa treatment.

3.4.1 Inhibition of nNOS by ARR17477 and 7-NI

Initial *ex vivo* studies confirmed that both ARR17477 and 7-NI could reduce nNOS activity in the striatum and cerebellum at 1 h following systemic administration. ARR17477 caused increasing inhibition with escalating doses, achieving a maximum reduction in nNOS activity at 12 mg/kg (56-60 %). Such inhibition of nNOS is consistent with Zhang *et al.* (1996), O'Neill *et al.* (2000) and Reif *et al.* (2000) who showed a significant reduction of nNOS activity following ARR17477 (1- 10 mg/kg i.v.) treatment at 2-3 h in rat cortex and cerebellum, although higher levels of inhibition were seen under these circumstances presumably owing to i.v. administration. Only Johansson *et al.* (1999) have employed ARR17477 subcutaneously at comparable doses, showing positive behavioural effects attributed to nNOS inhibition at 0.5-5.0 mg/kg, but no *ex vivo* investigation was included. Thus the present study further verifies the utilisation of ARR17477 by a subcutaneous route of administration coupled with evidence for nNOS inhibition at an earlier (1hr) time point than previously described.

The reduction of nNOS activity by 7-NI was maximal at 12.5-25.0 mg/kg and lessened as the dose was increased to 50 mg/kg i.p., which may be indicative of non-specific activity at the highest dose (see later discussion). Lack of a concentration effect on nNOS inhibition with increasing dose of 7-NI may also be explained by a reduction in solubility of the drug with increasing dose, whereas ARR17477 demonstrated full solubility and a dose-response effect. The findings corroborate work by Moore *et al.* (1993b) who showed 7-NI (25 mg/kg i.p.) produces significant inhibition of nNOS in mouse cerebellum at 15 min without affecting mean arterial blood pressure, and Mackenzie *et al.* (1994) who found 7-NI (30 mg/kg i.p.) significantly reduced nNOS activity in rat cerebellum, striatum and cortex within 30 min, lasting for at least 4hrs. nNOS inhibition may be underestimated by the *ex vivo* as some dissociation of the inhibitor may have occurred during the assay, especially in the case of 7-NI which has reversible, relatively short-lasting effects (Moore & Bland-Ward, 1996). Indeed Salter *et al.* (1995), showed that there is a decrease in accuracy of a NOS assay in reflecting *in vivo* NOS activity as incubation time is increased, especially where inhibitors dissociate within a short time-frame. Again these discrepancies, suggest inhibitory activity would have been greater in reality.

In conclusion the doses of nNOS inhibitors used in the *ex vivo* rat studies, specifically ARR17477 (3, 6 and 12 mg/kg s.c.) and 7-NI (12.5, 25 and 50 mg/kg ip), were sufficient to cause a reduction in central nNOS activity, justifying the doses used in further studies.

3.4.2 The effect of nNOS inhibition on L-dopa-induced AIMS expression

Neither of the nNOS inhibitors ARR17477 or 7-NI, administered at brain penetrable doses to reduce nNOS activity, significantly attenuated L-dopa induced axial, limb, orolingual or locomotive AIMs expression in the L-dopa-primed 6-OHDA-lesioned rat. Indeed, contrary to the original hypothesis, 7-NI in combination with L-dopa extended the duration of ALO AIM's expression.

The extension in duration of L-dopa-induced ALO AIMs observed in these studies following 7-NI (50 mg/kg) may be accounted for by non-nitrergic effects of the nNOS inhibitor as they were not observed following ARR17477 treatment. Non-nitrergic effects are further supported by the *ex vivo* data showing a small decrease in nNOS inhibition by 7-NI as the dose was increased from 25 mg/kg to 50 mg/kg. In addition to its nNOS activity there is evidence to suggest that 7-NI is also a potent MAOB inhibitor *in vitro*, where Ki = 4 µM, and also *in vivo* (Castagnoli *et al.*, 1997; Di Monte *et al.*, 1997; Desvignes *et al.*, 1999; Thomas *et al.*, 2008). 7-NI administration alone was seen to induce some mild AIMs in 6-OHDA-lesioned rats, above those of ARR17477 alone (although not significantly so) suggesting an inbalance in dopaminergic activity occurred in the 7-NI group which may have been caused by MAOB inhibitory effects. As well as inhibiting any upregulation of nNOS resulting from exposure to L-dopa, 7-NI could also prolong the duration of L-dopa effects by inhibiting the enzymatic breakdown of dopamine by MAOB (LeWitt, 1992). MAOB inhibition would account for an increased duration of L-dopa activity in the presence of 7-NI and hence the accompanying AIMS, without a worsening in their severity, as was indeed seen for axial, limb, orolingual and ALO AIMs at later time points. It should be noted that ARR17477 shows a mild interaction with MAOB *in vitro* (see Appendix, **Table 0-1**) although whether its activity is inhibitory or excitatory and would hold true *in vivo* is unknown. MAOB inhibitory effects may therefore not fully explain these differences between the duration of AIMs expression by L-dopa in combination with 7-NI and with ARR17477.

It has also been reported that 7-NI may not be as selective for nNOS *in vivo* as originally described by Moore and colleagues (1993a). Zagvazdin *et al.* (1996) showed 7-NI (50 mg/kg) administration to cause a significant increase in mean arterial blood pressure suggesting eNOS activity may also be affected. *In vitro* data also support the relative lack of isoform selectivity with Babbedge *et al.* (1993) showing little difference in selectivity for nNOS over eNOS, and Vallance and Leiper (2002) reporting that 7-NI shows only 1.4-fold selectivity for nNOS over eNOS whereas ARR17477 shows 23-fold selectivity for nNOS over eNOS. Therefore at the highest dose of 7-NI where the duration of ALO AIMs is most extended compared to activity following L-dopa alone eNOS related effects could certainly account for this difference. Notably Padovan-Neto *et al.* (2009) did not report any increase in AIMs with 7-NI in combination with L-dopa and this may be due to their utilisation of a lower maximal dose of the nNOS inhibitor of only 30 mg/kg. The regulation of vascular smooth muscle by eNOS enables circulatory modulation of brain microvessels and consequently the inhibition of eNOS by 7-NI could result in vasoconstriction (Rees *et al.*, 1989) which may interfere with the rate of L-dopa clearance hence prolonging the drug's effects. Indeed, Kelly *et al.* (1995) showed widespread reductions in regional

cerebral blood flow by 7NI (25 & 50 mg/kg). However further investigations would be necessary to confirm an effect of 7NI on dopaminergic pharmacokinetics as no data exist in the literature concerning eNOS-induced vasoconstriction and L-dopa clearance.

It is noteworthy that although ARR17477 is more potent and nNOS-selective than 7-NI, it is still not ideal as an nNOS inhibitor showing a maximum 100-fold selectivity for nNOS over eNOS in the published literature (Reif *et al.* 2000; Johansson et al., 1999). However it is the best nNOS inhibitor available to date that can be systemically administered.

Why do nNOS inhibitors not reduce the expression of dyskinesia in this study? Dyskinesia was undoubtedly present in all groups of animals used for these studies as demonstrated by the moderate to severe AIMs scores evident across the groups and dyskinesia clearly has the potential to be relieved as seen in the characterisation studies of Chapter 2 (section 2.3.2.3) where MK-801, 8-OHDPAT and amantadine all reduce expression of L-dopa-induced AIMs. Furthermore the AIMs model employed as established by Cenci and colleagues (1998) has been extensively characterised in the published literature and drugs reported to cause a reduction in dyskinesia in the clinic such as amantadine and clozapine have also been confirmed effective in this model (Lundblad *et al.*, 2002; Dekundy *et al.*, 2007).

The most obvious explanation for the lack of efficacy in reducing AIMs by nNOS inhibition is that increased nNOS activity in itself is not a key factor underlying the expression of dyskinetic behaviour or perhaps the modulation of nNOS alone is insufficient to reduce dyskinesia in this model. However, this finding is in contrast to the finding of Padovan-Neto *et al.* (2009) who reported that 7-NI (30 mg/kg i.p.) reduced ALO and locomotive AIMs scores in the 6-OHDA-lesioned rat model. However there were a number of differences between these two studies – Padovan-Neto *et al.* (2009) initially primed animals with an exceptionally high dose of L-dopa (100 mg/kg p.o.), far beyond that required to reverse unilateral asymmetry and also routinely used to prime and induce AIMs (Cenci *et al.*, 1998; Westin *et al.*, 2001; Picconi *et al.*, 2003). This difference in dosage could have had various implications on underlying molecular changes occurring within the brain during the priming process, as supported by the fact that even naïve primates may develop dyskinesia following repeated high dose L-dopa administration (Pearce *et al.*, 2001).

Additionally the dose of L-dopa used by Padovan-Neto *et al.* (2009) to express AIMs was also considerably high (30 mg/kg p.o.) compared to the dose used for the studies presented in this chapter (6.25 mg/kg i.p.), and in comparison with the literature (Cenci *et al.*, 1998; Putterman *et al.*, 2007; Dupre *et al.*, 2008; Monville *et al.*, 2009). Indeed such higher doses of L-dopa greatly enhance rotational behaviour to the extent that they may obscure dyskinesia expressed in the trunk and limbs (Marin *et al.*, 2006). It is therefore possible that some dyskinesia may have gone unnoticed in these experiments. The difference in route of administration of L-dopa must be borne in mind, as oral bioavailability of L-dopa is lower than for systemic administration (Rose *et al.*, 1993; Bredberg *et al.*, 1994), although this discrepency does not fully explain the need for employing such high doses. Additionally, AIMS were only scored at 60 and 120 min after dopaminergic treatment which may also have concealed any differences

in behaviour occurring at time points in between these observations. The findings presented within this chapter are in keeping with the AIMs model as developed by Cenci and collegues (1998), and pharmacologically relevant doses of dopaminergic drugs used to control motor symptoms in PD patients.

These studies show that nNOS inhibition by ARR17477 or 7-NI does not reduce L-dopa-induced dyskinesia in the 6-OHDA-lesioned rat model.

3.4.3 The effect of nNOS inhibition on ropinirole-induced AIMS expression

As expected, the dopamine agonist ropinirole induced less severe dyskinesia overall, as measured by AIMs, compared to L-dopa. This supports the literature where ropinirole is described to induce more naturalistic behaviours without stereotopies, whilst L-dopa causes stereotypic behaviour and compulsive grooming in the 6-OHDA-lesioned rat (Eden *et al.*, 1991; Ravenscroft *et al.*, 2004). However, only descriptions of behaviour were published and AIMs were not measured *per se*. The present finding corroborates a more recent study showing ropinirole to cause a significant reduction in both overall duration and total ALO AIMs expression compared to L-dopa (Papathanou *et al.*, 2011). As mentioned previously, some evidence suggests that relatively lower dyskinesia expression may occur after switching from L-dopa to dopamine agonists in animal models and PD patients further validating the observed difference, although it is uncertain whether benefits are long-lasting (Kapoon *et al.*, 1989; Facca & Sanchez-Ramos, 1996; Hadj Tahar *et al.*, 2000; Jackson *et al.*, 2007).

No significant reduction in AIMs subcategories by the nNOS inhibitors ARR17477 or 7-NI was observed when animals were treated with ropinirole in place of L-dopa. Similar to the effects seen on L-dopa-induced AIMS, 7-NI in combination with ropinirole increased the duration of ALO AIMs in addition to increasing the duration of axial and locomotive AIMs expression. The overall total ALO AIMs expression was also increased by 7-NI administration.

7-NI (50 mg/kg) significantly extended the overall duration of both ropinirole-induced axial and ALO AIMs activity by 75 min and total AIMS scores were also significantly higher for axial, limb and ALO AIMs meanwhile L-dopa-induced ALO AIMS were significantly extended by only 45 min without affecting totals or other AIMs subcategories. The general rationale for nNOS inhibitors not reducing ropinirole-induced AIMs expression are equally valid as for L-dopa effects as described in section 3.4.2. In particular ropinirole-induced AIMs extension by 7-NI may be related to its MAOB associated activity, especially considering no such increase in duration of AIMs was induced by ARR17477. However MAOB effects are unexpected with a dopamine agonist acting post-synaptically. A possible explanation is that MAOB inhibition could enhance levels of any remaining endogenous dopamine which would act on both D1 and D2 receptors. There is evidence to suggest that a small amount of D1 activity coupled with D2-specific agonism results in synergistic effects potentiating dopamine levels and associated behaviour in lesioned-rats (Arnt & Perregaard, 1987). The interaction between dopamine receptors could justify why

the effect of 7-NI on AIMs appears more exaggerated with ropinirole (D2 selective) than L-dopa (D1 & D2). Additionally this phenomenon may relate to eNOS inhibition as already described in terms of the extension of the L-dopa response (see section 3.4.2).

Furthermore, the shorter duration of AIMs expression in the 6-OHDA-lesioned L-dopa-primed rat observed following ropinirole compared to L-dopa, as corroborated by Papathanou *et al.* (2011), would afford an increased period over which to potentially extend AIMs. This difference may provide a practical explanation as to why 7-NI in combination with ropinirole appeared to have a greater impact on dyskinesia expression both prolonging the duration over a wider time frame and also affecting more subtypes of overall AIMS than 7-NI in combination with L-dopa. Had AIMs been assessed over a longer time period following L-dopa treatment then less differences may have been apparent. This observation may also be dose-dependent and were a higher dose of ropinirole chosen it is possible that effects would not be dissimilar between the two types of dopaminergic agents.

These studies show that nNOS inhibition by ARR17477 or 7-NI does not reduce ropinirole-induced dyskinesia in the 6-OHDA-lesioned rat model.

3.4.4 Conclusion

In summary, no significantly beneficial effect of nNOS inhibition on L-dopa-or ropinirole-induced AIMs expression has been demonstrated in L-dopa-primed 6-OHDA-lesioned rats within the studies discussed so far. Neither ARR17477 nor 7-NI significantly attenuated AIMs expression by dopaminergic drugs, and indeed the highest dose of 7-NI was shown to extend the duration of AIMs expression in combination with L-dopa and ropinirole, which is likely to be due to non-nNOS-specific effects of 7-NI.

There may be key differences between mechanisms underlying the expression of AIMs by single acute challenges with dopaminergic agents and those changes occurring during the initial priming process where dyskinesia is induced in otherwise drug naïve animals. It is important to determine whether nNOS inhibitors may be effective in reducing or even preventing AIMs if administered in combination with dopaminergic drugs from the outset. The next chapter will investigate the role of nNOS inhibitors within this context.

Chapter 4 : The effects of nNOS inhibitor treatment on priming for AIMs in 6-OHDA-lesioned rats

4.1 Introduction

In the preceding chapter the inhibition of nNOS did not reduce the expression of AIMs generated by L-dopa or ropinirole in L-dopa-primed 6-OHDA-lesioned rats. This finding suggests that neuronal nitric oxide may not be a critical factor underlying the expression of pre-established dyskinesia in rats following long-term dopaminergic treatment. Prior to nNOS inhibitor administration the animals employed within these previous studies had already received chronic treatment with L-dopa to induce behavioural changes, and accordingly the underlying molecular changes responsible for dyskinesia expression. By this stage irreversible adaptations may have taken place within the basal ganglia circuitry, which could no longer be impeded by nNOS inhibition, such as long-term changes in synaptic transmission (Belujon et al., 2010).

The manifestation of abnormal movements is considered a two-step process whereby 'expression' of dyskinetic behaviour is brought about by an induction, or so-called 'priming', phase (Brotchie, 2005). Thus, expression studies tend to mimic the later stages of PD therapeutics where dyskinesia is fully established and therefore exhibited with each subsequent dose of dopaminergic medication. The molecular changes underlying the induction of dyskinesia may well differ from those responsible for the execution of involuntary movements (Jenner, 2008b). Additionally due to the large extent of dopamine neurone degeneration necessary for the 6-OHDA-lesioned rat to potentially demonstrate dyskinesia (Cenci, 2007), the model pathologically mimics advanced PD when it may be markedly more challenging to influence molecular changes. The mechanisms underlying priming are yet to be fully elucidated but a variety of long-term pre- and post-synaptic changes undoubtedly accompany the appearance of dyskinesia both in animal models and in patients with PD, as described in detail in Chapter 1 (Cenci & Lundblad, 2006; Jenner, 2008b; Santini *et al.*, 2008).

The malfunction of corticostriatal plasticity (modified transmission efficacy occurring at synapses), has been closely linked to the manifestation of dyskinesia particularly due to its persistent nature and a correlation with severity of abnormal movements (Calabresi *et al.*, 2000; Picconi *et al.*, 2003; Prescott *et al.*, 2008). Furthermore, nitric oxide (NO) acts as a critical messenger capable of initiating molecular alterations leading to changes in synaptic plasticity such as long-term potentiation and long-term depression (LTP/LTD) in the CNS and more specifically the striatum (Calabresi *et al.*, 1999; Prast & Philippu, 2001; West *et al.*, 2002). Aside from the extent of striatal dopamine loss, the onset of dyskinesia is inextricably related to the use of dopaminergic medication (Crossman, 1990), thus intervention occurring before these long-term alterations or synaptic changes are induced could potentially prevent dyskinesia.

Implications of timing raise the question, if drug naïve 6-OHDA-lesioned rats were co-administered nNOS inhibitors from first exposure to dopaminergic treatment, is it possible that the priming phenomenon (and hence the subsequent expression of dyskinesia) could be disrupted?

4.1.1 Hypothesis

It is hypothesised that inhibition of nNOS will prevent the induction of dyskinesia by dopaminergic drug treatment in 6-OHDA-lesioned rats.

4.1.2 Aims

The aim of the studies reported in this chapter was to determine whether nNOS inhibitors can prevent the induction of dyskinesia (priming) following administration of dopaminergic treatment to drug naive 6-OHDA-lesioned rats, more specifically to investigate;

1. The effect of chronic treatment with the selective and irreversible nNOS inhibitor ARR17477 on nNOS activity at a range of doses *ex vivo*.

2. The effect of the nNOS inhibitors ARR17477 and 7-NI on the induction of AIMs by chronic L-dopa administration in 6-OHDA-lesioned rats.

3. The effect of the nNOS inhibitors ARR17477 and 7-NI on the induction of AIMs by chronic ropinirole administration in 6-OHDA-lesioned rats.

4.2 Materials and methods

4.2.1 Introduction

These studies aimed to investigate the effect of chronic administration of nNOS inhibitors on the priming for AIMs by L-dopa and ropinirole in 6-OHDA-lesioned rats. Although 7-NI has already been used widely in chronic treatment studies it was first necessary to establish the dose of ARR17477 required in order to inhibit nNOS for the chronic *in vivo* studies. Subsequently the effect of chronic administration of nNOS inhibitors was investigated on dopaminergic-mediated induction of AIMs, in drug-naïve 6-OHDA-lesioned rats. AIMs were assessed regularly throughout the course of long term treatment to ascertain the effects of nNOS inhibition. The methods for these studies are described below.

4.2.2 Animals

For all experiments male Wistar rats (200-250 g; Harlan, UK or B & K, UK) were housed 2-3 per cage in the Biological Service Unit, King's College London. Room temperatures were maintained at 19-21 ˚C at 55 % humidity with a 12 h light-dark cycle and animals had free access to pelleted food and water, as described in section 2.2.2. Experiments were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986 under Home Office project licence no. 70/6019 or 70/6898.

4.2.3 Determination of the dose of nNOS inhibitor

4.2.3.1 7-NI

A dose of 7-NI (25 mg/kg dissolved in DMSO:saline 0.9 %, 50:50, i.p.) was chosen based on previous chronic studies performed in these laboratories and was in agreement with the published literature, (Mackenzie *et al.*, 1994; Przedborski *et al.*, 1996; Bush & Pollack, 2001; Li *et al.*, 2002). This same dose (25 mg/kg i.p.) was also employed in the *ex vivo* and acute study of effects of nNOS inhibitors on dyskinesia expression (See Chapter 3, section 3.2.3). Taking into account the reversible nature of the inhibitor and its short half life (Moore *et al.*, 1993b) as well as its use in numerous chronic studies to date, there was no concern over the effects of long-term 7-NI treatment in rats, hence no further *ex vivo* was warranted with this drug. Additionally studies have also shown there to be no adverse effects of dosing repeatedly with 7-NI at a similar dose (Przedborski *et al.*, 1996; Li *et al.*, 2002; Wangensteen *et al.*, 2006).

4.2.3.2 ARR17477

It was desirable to reduce the dose of ARR17477 as far as possible from that employed in the acute studies of Chapter 3, to eliminate non-specific drug effects owing to drug accumulation during chronic treatment, due to its long lasting inhibition and irreversible nature (Zhang *et al.*, 1996; Reif *et al.*, 2000). These could include activity at adrenergic receptors, vesicular monoamine, norepinephrine or dopamine transporters (see Appendix, Table 0-1), or effects on eNOS activity.

Therefore to determine the chronic effect of the nNOS inhibitor ARR17477 in rat brain, enzyme activity was assessed in rats by measuring the conversion of L-arginine to L-citrulline *ex vivo* following chronic systemic administration at a range of doses. The dose of the nNOS inhibitor ARR17477 to be used in

these studies was determined in experiments measuring the inhibition of nNOS activity *ex vivo* following chronic systemic administration.

4.2.3.2.1 Ex vivo determination of ARR17477 dose

Naïve male Wistar rats (n=4/group; 200-250 g) were treated daily with ARR17477 (0.5, 1, 3 or 6 mg/kg s.c.) or vehicle (saline 0.9 %; 1 ml/kg s.c.) for four consecutive days and culled by decapitation at 12 h after the final dose. These doses were chosen based on those selected for acute studies (see Chapter 3), taking into consideration the implications of irreversible binding properties of ARR17477 (personal communication with R.Silverman, Northwestern University, Chicago) on a chronic treatment regime. The cerebellum was dissected out, snap frozen and stored at -70 ˚C for measurement of nNOS activity by radioenzymatic assay.

In order to confirm nNOS inhibition at an earlier time point the *ex vivo* study was repeated with animals treated with ARR17477 1 mg/kg s.c. or vehicle (saline 0.9 %; 1 ml/kg s.c.) for four consecutive days and then culled by decapitation at a 1 h time point after the final dose. Tissue was then dissected out as described above.

4.2.3.2.2 Radioenzymatic measurement of NOS activity

nNOS activity was determined in brain homogenates by measuring enzymatic conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline as fully described in section 2.6.1. Briefly homogenised tissue samples were centrifuged (as detailed in section 2.6.1.2), and supernatants transferred to a 96-well plate in triplicate. Boiled supernatant was used as a negative control. A pre-prepared reaction mixture containing L-[2,3,4-^{3}H] arginine monohydrochloride (1 mCi/ml; 16.7 µM) and $CaCl_2$ (6 mM) was added (30 µl/well) and the plates incubated for 1 h at 30 °C. The reaction was terminated and the reaction mixture further processed to measure L-[^{3}H]-citrulline formation, by the addition of resin and subsequent filtration of the samples as described in section 2.6.1.2. L-[^{3}H]-citrulline was determined in a Beta-liquid scintiallation counter (see section 2.6.1.2) whilst protein content of samples was quantified with a NanoDrop spectrophotometer (see 2.6.1.3). Data were analysed as described in section 2.6.1.4 expressing nNOS activity as L-[^{3}H]-citrulline formation per mg of protein per hour.

4.2.3.2.3 ARR17477 results

ARR17477 lowered nNOS activity in a dose-related manner with a significant reduction of 56, 61 and 73 % following doses of 1, 3 and 6 mg/kg s.c. respectively in the cerebellum at 12 h following 4 days of chronic treatment (**Figure 4-1**). A further ex vivo study of ARR17477 (1 mg/kg s.c.) dosed for 4 consecutive days showed a 95 % reduction in nNOS activity at 1 h (**Figure 4-2**). This time point would coincide with peak plasma levels of dopaminergic treatment and therefore ARR17477 (1 mg/kg s.c.) was chosen to be used in subsequent studies. Due to technical issues only data for cerebellar tissue were attained.

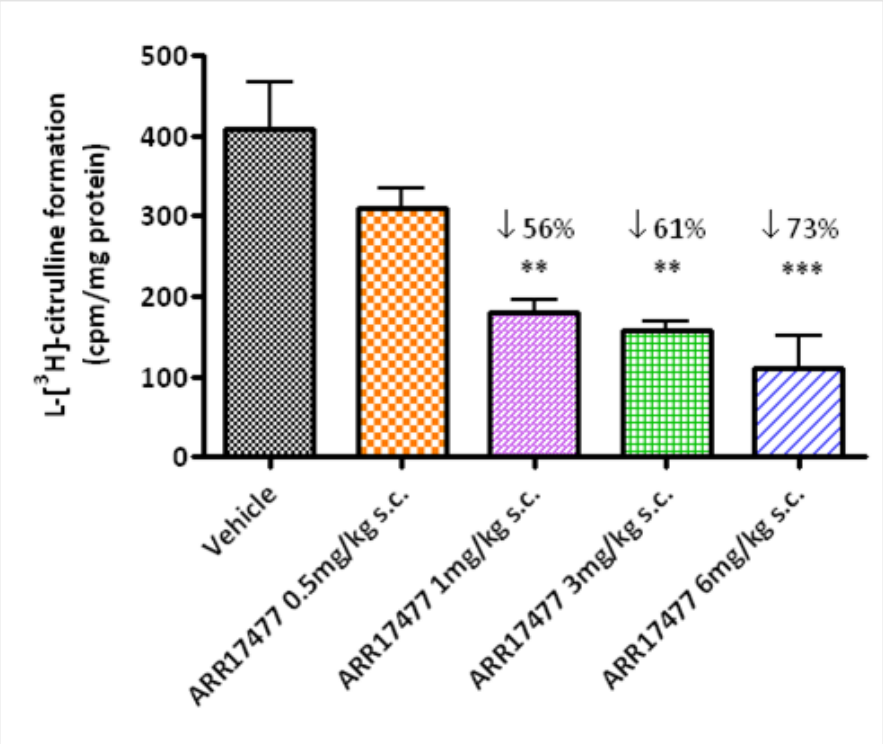

Figure 4-1 Radioenzymatic measurement of the effect of repeated ARR17477 (0.5, 1, 3 or 6 mg/kg s.c.) treatment on nNOS activity in cerebellum. Naïve rats were treated with ARR17477 or vehicle (0.9 % saline s.c.) for four consecutive days and culled by decapitation at 12 h after the final dose. nNOS activity is displayed as L-[3H]-citrulline formation (cpm/mg protein); Data are presented as means ± SEM (n=4/group); **P<0.01 *** P<0.001 compared to vehicle treatment. Data were analysed by one-way ANOVA followed by Newman-Keuls post hoc test.

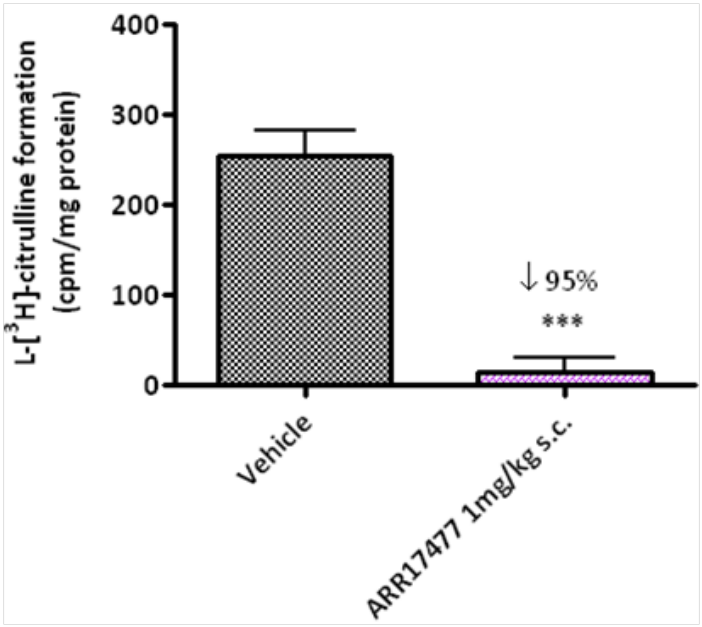

Figure 4-2 Radioenzymatic measurement of the effect of repeated ARR17477 (1 mg/kg s.c.) treatment on nNOS activity in cerebellum. Naïve rats were treated with ARR17477 or vehicle (0.9 % saline s.c.) for four consecutive days and culled by decapitation at 1 h after the final dose. nNOS activity is displayed as L-[3H]-citrulline formation (cpm/mg protein); Data are presented as means ± SEM (n=4/group); *** P<0.001 compared to vehicle treatment. Data wereanalysed by t-test.

4.2.4 Behavioural Studies

4.2.4.1 Unilateral 6-OHDA lesion

Male Wistar rats (see section 4.2.2) maintained under general anaesthesia (2-3 % isofluorane in 95 % O_2 and 5 % CO_2) were unilaterally lesioned with 6-OHDA in the left MFB using standard stereotaxic techniques, as described fully in section 2.2.3. In brief, 6-OHDA hydrochloride (8 µg free base in 4 µl 0.9 % saline containing 0.05 % ascorbic acid) was injected at a rate of 1 µl per minute into the medial forebrain bundle (according to the coordinates of Paxinos and Watson, 1986; A-P:-2.6 mm, M-L: +2.0 mm, V:-8.8 mm as measured from bregma). The needle was left in place for 4 min to allow for the 6-OHDA to diffuse into the surrounding region and then slowly withdrawn before cleaning and suturing the wound. Animals were cared for post-operatively as described in section 2.2.4.

Three-four weeks after surgery rats were treated with amphetamine sulphate (2.5 mg/kg i.p. in 0.9 % saline) and rotational behaviour was monitored as described in section 2.3.1. Only rats exhibiting 7 or more ipsilateral turns per minute at peak activity were used for further studies.

4.2.4.2 Chronic drug challenges in combination with nNOS inhibitors

Untreated 6-OHDA-lesioned rats were divided into seven treatment groups (n=8-10/group), as shown in **Table 4-1**, each balanced for mean total rotations following amphetamine screening as described in 4.2.4.1. Preliminary studies were performed to establish whether ARR17477/7-NI induced dyskinesia with chronic treatment (Groups 1a & 1b, **Table 4-1**). Subsequently priming studies with L-dopa or ropinirole alone or in combination with nNOS inhibitors were carried out (Groups 2-7, **Table 4-1**). These studies employed a staggered design where representative animals from each treatment group were assessed for AIMs daily.

Animals (Groups 2-7) were treated once daily with nNOS inhibitors ARR17477 (1 mg/kg in saline 0.9 % s.c.) or 7-NI (25 mg/kg in DMSO: saline 0.9 %, 50:50 i.p), or vehicle (1 ml/kg; saline 0.9 % s.c. or DMSO: saline, 0.9 %, 50:50 i.p.) for 3 days prior to co-administration of nNOS inhibitors with L-dopa methyl ester (6.25 mg/kg free base + benserazide 15 mg/kg, in saline 0.9 %, i.p.) or ropinirole (0.2 mg/kg, in saline 0.9 %, i.p.) daily for 22 days. Group 1 animals received nNOS inhibitors alone for the pre-treatment and treatment phases amounting to 25 days in total. See **Table 4-1** for individual treatment groups and course of drug administration.

At the end of the chronic treatment phase following a 1 week drug washout animals were challenged acutely with L-dopa methyl ester (6.25 mg/kg free base + benserazide 15 mg/kg i.p.) or ropinirole (0.2 mg/kg i.p.) on two separate occasions 1 week apart.

4.2.4.3 AIMs assessment

Dyskinesia was assessed based on the observation of four subtypes of AIM's; locomotive, axial, limb and orolingual (originally described by Cenci et al., 1998), each rated on a scale of 0-4 dependent on frequency and intensity (see section 2.3.2.2). AIMs were assessed one day prior to the start of nNOS pretreatment for habituation to AIMs cages (Day -3; baseline$_1$ 'B_1'), once on the first day of nNOS

pretreatment (Day -2; baseline$_2$ 'B_2') and then every third day starting from Day 1-22 of chronic treatment. AIMs were also assessed in the two final acute challenges described above (see **Figure 4-3**).

On each assessment day rats were placed in transparent cages (set up as shown in Figure 2.5, Chapter 2) one hour before drug treatment commenced to enable acclimatisation (as described in section 2.3.2.2). Baseline AIMs for each session were scored 20 min and 5 min prior to dosing as described in section 2.3.2.2. Following drug treatment AIMs were assessed for 5 min every 15 min for a total of 210 min in the L-dopa priming studies and 180 min in the ropinirole priming studies.

Table 4-1 AIMs assessment treatment groups and drug administration during initial habituation (day -3), pre-treatment (days -2-0), treatment (days 1-22) and final acute challenges. Groups 1a&b were nNOS inhibitor controls, 2-4 were nNOS inhibitor + L-dopa treatment groups and 5-7 were nNOS inhibitor + ropinirole treatment groups. L-dopa was always administered with benserazide (15 mg/kg i.p.).

Group	n	B_1 Day -3	Pre-treatment B_2 Days -2-0	Treatment Days 1-22	Final acute challenges
1a 1b	4 4	- -	ARR17477 (1 mg/kg s.c.) 7-NI (25 mg/kg i.p.)	ARR17477 (1 mg/kg s.c) 7-NI (25 mg/kg i.p.)	- -
2	10	-	Vehicle	L-dopa (6.25 mg/kg i.p.) + vehicle	L-dopa (6.25 mg/kg i.p.)
3	10	-	ARR17477 (1 mg/kg s.c)	L-dopa (6.25 mg/kg i.p.) + ARR17477 (1 mg/kg s.c)	L-dopa (6.25 mg/kg i.p.)
4	10	-	7-NI (25 mg/kg i.p.)	L-dopa (6.25 mg/kg i.p.) + 7-NI (25 mg/kg i.p.)	L-dopa (6.25 mg/kg i.p.)
5	10	-	Vehicle	ropinirole (0.2 mg/kg i.p.) + vehicle	ropinirole (0.2 mg/kg i.p.)
6	10	-	ARR17477 (1 mg/kg s.c)	ropinirole (0.2 mg/kg i.p.) + ARR17477 (1 mg/kg s.c)	ropinirole (0.2 mg/kg i.p.)
7	10	-	7-NI (25 mg/kg i.p.)	ropinirole (0.2 mg/kg i.p.) + 7-NI (25 mg/kg i.p.)	ropinirole (0.2 mg/kg i.p.)

4.2.5 Data and statistical analysis

Total and peak AIMs scores, and duration of AIMs activity were plotted as medians, throughout the chronic treatment phase, using data collected from individual test days. Any animals showing <90 % dopaminergic cell loss in SNpc at the lesioned compared to non-lesioned hemishphere, determined post-behavioural experiments by TH immunohistochemistry (See Chapter 2; section 2.6.2), were excluded from data analysis. Total AIMs scores for each assessment day were calculated by AUC (Graphpad Prism version 5.0) using the trapezoid method where each successive 15 min was labelled as a single time-bin and peak score was taken as the maximum AIMs score achieved per 15 min. The duration of AIMs was calculated taking into account all score periods gaining above 0 and in the case of orolingual AIMs included those gaining above 1. Chronic treatment data were analysed by 2-way ANOVA

and Friedman's test, and additionally Kruskal-Wallis test followed by Dunn's post hoc test where appropriate. Summed data were also plotted for the full priming period using AUC (Graphpad Prism version 5.0) of 22 day total and peak AIMs scores, and duration of AIMs activity. Additionally final challenge data were plotted as a mean of the two final acute L-dopa challenges for total and peak AIMs scores, and duration of AIMs activity. Kruskall Wallis tests were used to compare the effect of treatment on summed AIMs scores for the priming period and also the subsequent L-dopa challenges. In all cases statistical significance was set at $p<0.05$ and analyses were carried out in Graphpad Prism 5. Additionally a 'trend' was described in the data where there was greater than a 75 % change from vehicle treated animals.

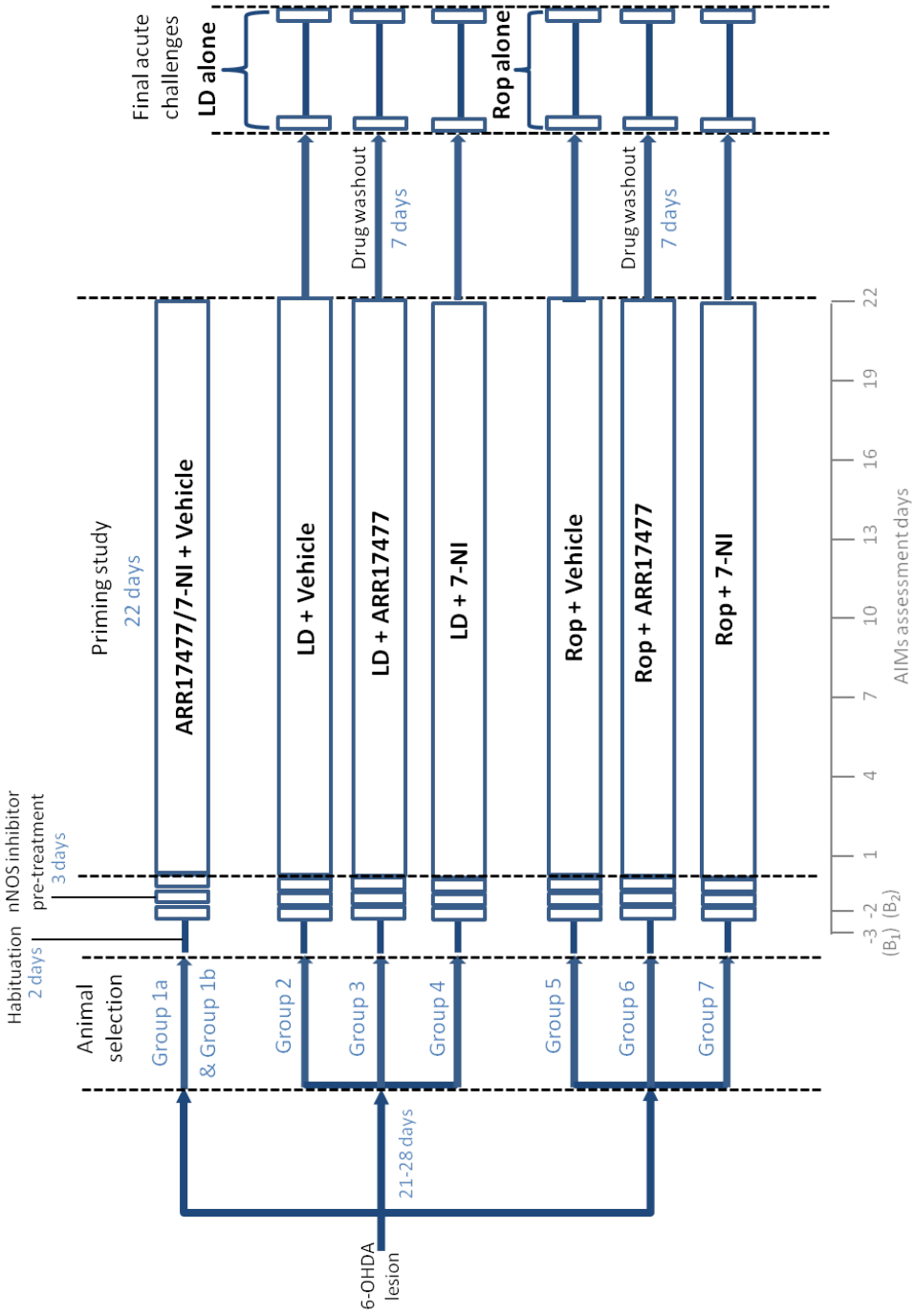

Figure 4-3 Summary time-line of animal groups and priming treatments for chronic AIMs studies with L-dopa (LD; 6.25 mg/kg + benserazide 15 mg/kg i.p.), ropinirole (Rop; 0.2 mg/kg i.p.) or vehicle (saline 1 ml/kg i.p.) in combination with ARR17477 (1 mg/kg s.c.), 7-NI(25 mg/kg i.p.) or vehicle. (saline ,or DMSO: saline 50:50, 1 ml/kg s.c./i.p.).

4.3 Results

4.3.1 AIMs in 6-OHDA-lesioned rats primed with nNOS inhibitor alone

No significant effect of chronic treatment with ARR17477 or 7-NI on the induction of locomotive, axial, limb, orolingual or ALO AIMs (Two-way ANOVA and Friedman's test on total, peak and duration of AIMs; data not shown). Therefore the two groups were combined to give an nNOS inhibitor control group. There was no effect of chronic nNOS inhibitor treatment alone on any AIMs subcategory, as exemplified in **Figure 4-4** showing total locomotive or ALO AIMs scores throughout the 22 days (individual AIMs scores and peak or duration scores not shown).

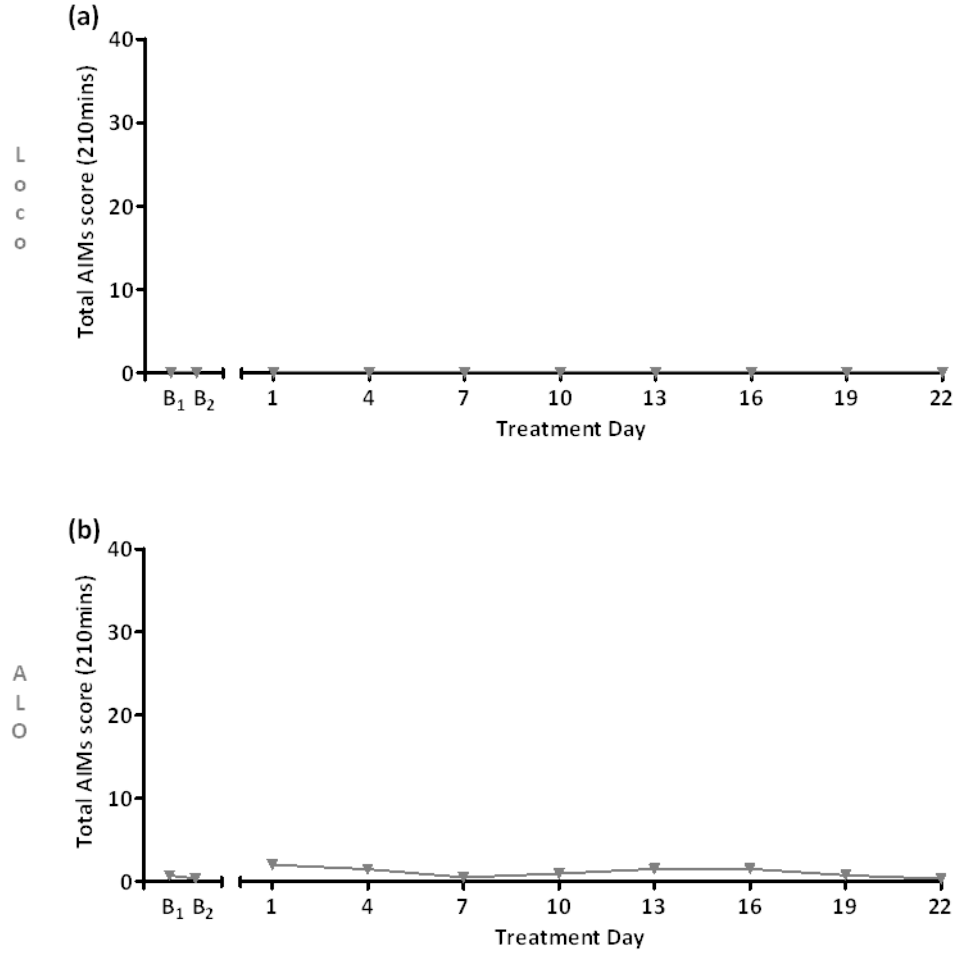

Figure 4-4 Locomotive (a) and ALO Total AIMs (b) following chronic treatment with ARR17477 or 7-NI. ARR17477 (ARR; 1 mg/kg s.c) or 7-NI (25 mg/kg i.p.) in 6-OHDA-lesioned rats. Data are combined for the two nNOS inhibitors and presented as medians (n=8); (a) locomotive, (b) ALO AIMs. Data were analysed by Friedman's test (B_1 :baseline 1 and B_2 :baseline 2).

4.3.2 Locomotive AIMs in 6-OHDA-lesioned rats chronically treated with L-dopa plus nNOS inhibitor

4.3.2.1 Vehicle + L-dopa chronic treatment

L-dopa alone induced locomotive AIMs scoring 2 on day 7 of chronic treatment increasing to a total of 5 by day 10 (**Figure 4-5a**). A maximum peak locomotive score of mild levels scoring 1 was achieved by day 10 continuing at a similar level through to day 22 (**Figure 4-5b**). The duration of locomotive AIMs activity increased from 25 min on day 7 to a maximal 90 min duration on day 10 and fluctuated between 25 and 80 min on assessment days thereafter (**Figure 4-5c**). As expected locomotive AIMs significantly changed over the priming period as measured by all three parameters, increasing from baseline levels prior to initiation of L-dopa treatment.

4.3.2.2 nNOS inhibitor + L-dopa chronic treatment

There was no significant effect of ARR17477 or 7-NI on L-dopa-induced locomotive AIMs as measured by total AIMs score (**Figure 4-5a**), peak (**Figure 4-5b**), or duration of activity (**Figure 4-5c**). Although ARR17477 induced mild AIMs peaking at 1 and lasting for 45 min at the earlier time point of day 4 as compared to the L-dopa alone where animals showed no AIMs at this stage, and locomotive AIMs tended to be reduced from day 7 onwards, these differences did not reach statistical significance (**Figure 4-5a**-**c**). The summed totals, and peaks graphs (**Figure 4-5a'**-**b'**) all showed a tendency for ARR17477 to reduce L-dopa-induced locomotive AIMs but again there was no statistical significance between the treatment groups.

4.3.2.3 L-dopa final acute challenges

Following chronic treatment with L-dopa alone, subsequent acute treatment with L-dopa alone induced a median total locomotive AIMs score of 5 (**Figure 4-6a**), with a peak score of 1 (**Figure 4-6b**) and a duration of 65 min (**Figure 4-6c**). There was no significant effect of chronic 7-NI plus L-dopa treatment on subsequent L-dopa-induced locomotive AIMs as measured by total or peak AIMs scores or durations of activity (**Figure 4-6a-c**). Chronic ARR17477 treatment tended to reduce total and peak AIMs scores and duration of activity, although these effects did not reach statistical significance (**Figure 4-6a-c**).

4.3.3 Locomotive AIMs in 6-OHDA-lesioned rats with ropinirole plus nNOS inhibitor

4.3.3.1 Vehicle + ropinirole

Ropinirole alone induced mild locomotive AIMs which significantly increased across the treatment period with a total score of 1.5 on day 13 steadily increasing to 4 by day 22 (**Figure 4-7a**). A peak score of 1.5 was achieved by day 22 (**Figure 4-7b**), and the average duration of activity increased from 15 min on day 13 to 45 min on day 22 (**Figure 4-7c**). Ropinirole produced locomotive AIMs of a similar severity level to L-dopa, but these were not expressed until a later time point and lasted for a shorter duration on each assessment day.

4.3.3.2 nNOS inhibitor + ropinirole

No significant effect of ARR17477 or 7-NI was observed on ropinirole-induced locomotive AIMs as measured by total AIMs (**Figure 4-7a**), peak scores (**Figure 4-7b**) or duration of activity (**Figure 4-7c**). 7-NI appeared to induce AIMs at an earlier time point than ropinirole alone although this effect was not significant. The summed totals, peaks and duration graphs (**Figure 4-7a'-c'**) further confirmed there was a trend for increased locomotive AIMs but no overall significant effect of treatment. There was also a trend for ARR17477 increasing summed total locomotive AIMs, although this effect did not reach statistical significance (**Figure 4-7a'**).

4.3.3.3 Ropinirole final acute challenges

Acute treatment with ropinirole alone induced an average total locomotive AIMs score of 4 (**Figure 4-8a**), with a peak of 1.75 (**Figure 4-8b**) and duration of activity of 45 min (**Figure 4-8c**) in animals previously chronically treated with ropinirole alone. There was no significant effect of ARR17477 or 7-NI plus ropinirole chronic treatment on subsequent ropinirole induced locomotive AIMs in terms of total scores, peaks or durations of activity (**Figure 4-8a-c**)

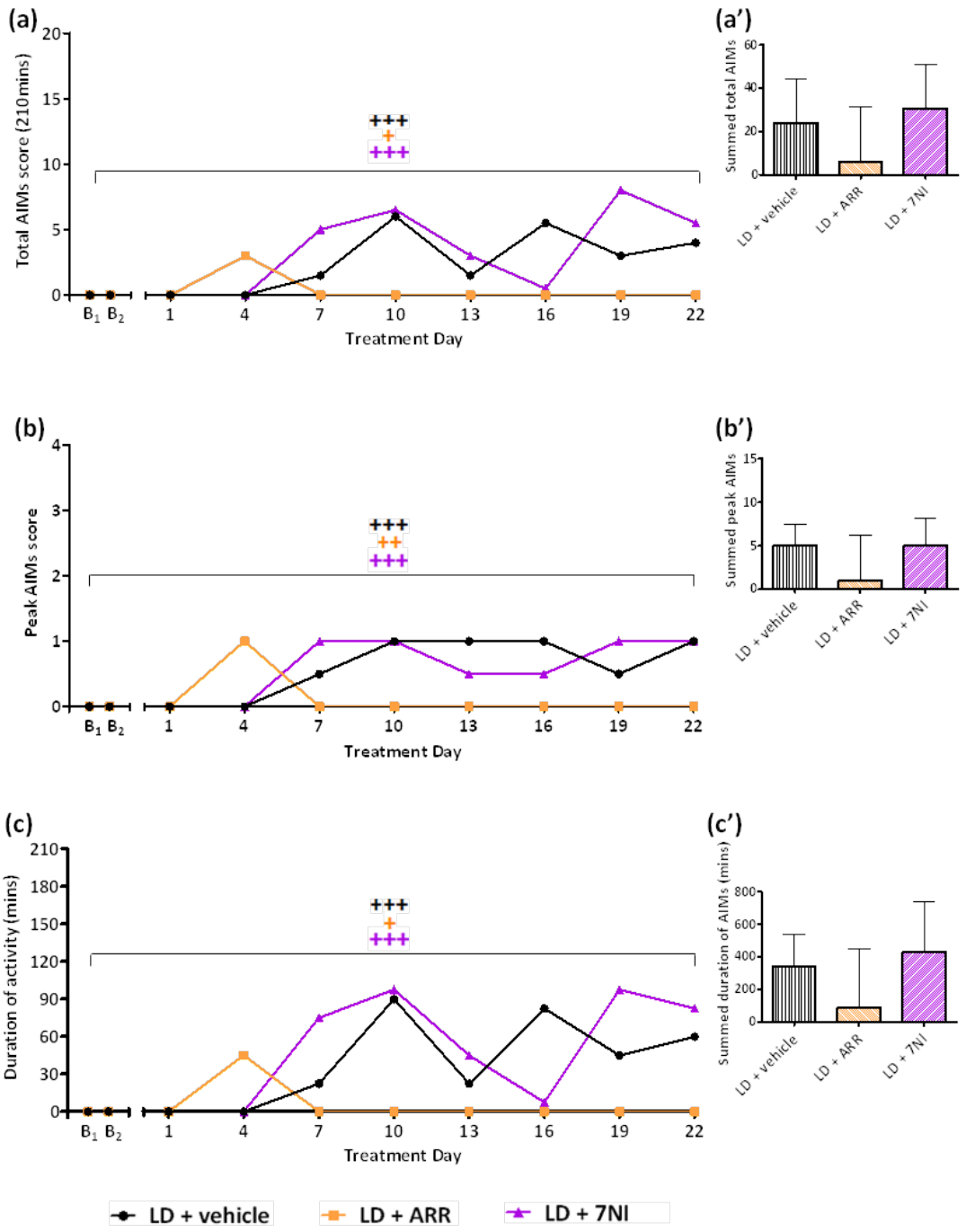

Figure 4-5 Locomotive AIMs following chronic treatment with ARR17477 or 7-NI plus L-dopa. ARR17477 (ARR; 1 mg/kg s.c), 7-NI (25 mg/kg i.p.) and L-dopa (LD; 6.25 mg/kg plus benserazide 15 mg/kg i.p.) in 6-OHDA-lesioned rats. Data are all presented as medians and also a'-c' as interquartile ranges, (n=9-10); **(a)** Total score, **(b)** Peak score, **(c)** Duration of activity and **(a'-c')** Summed data over all treatment days for graphs a-c respectively. +p<0.05, ++p<0.01, +++p<0.001 for time (colours used in (a-c) refer to key). Data (a-c) were analysed by 2-way-ANOVA and Friedman's test or (a'-c') by Kruskal-Wallis test (B_1 :baseline 1 and B_2 :baseline 2).

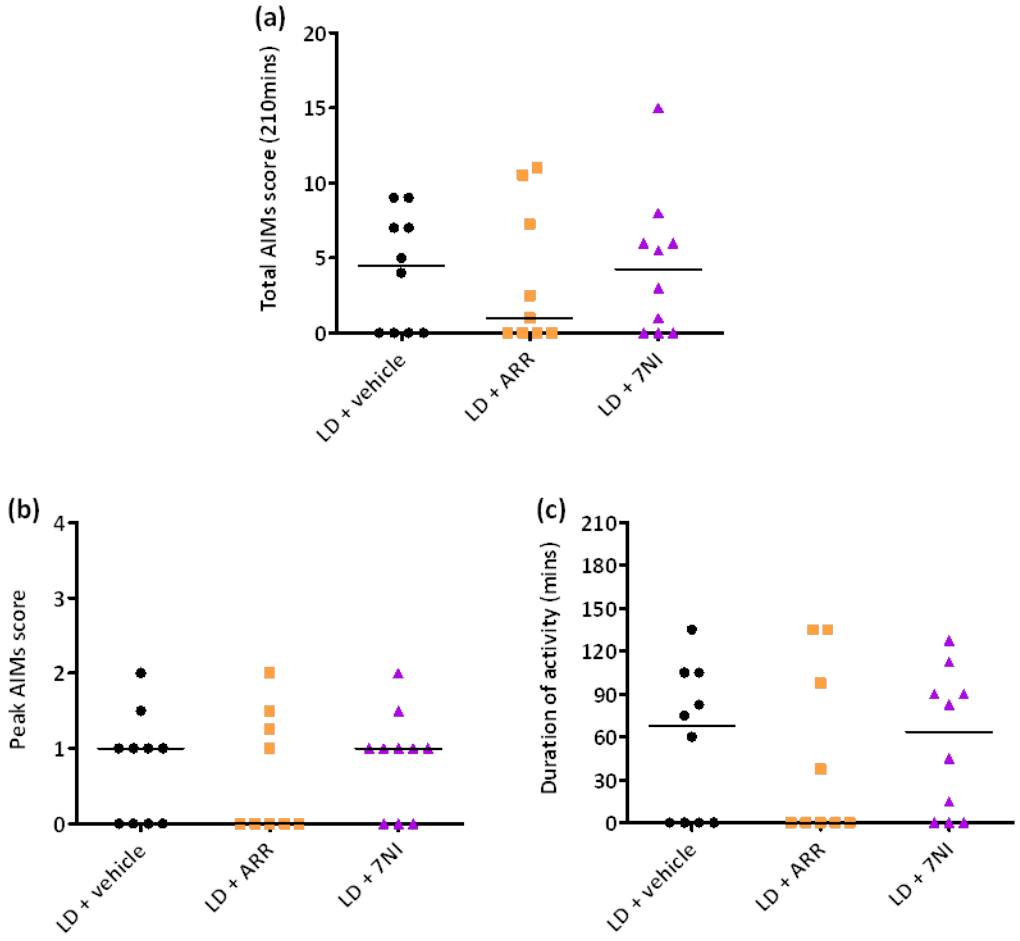

Figure 4-6 Locomotive AIMs following final L-dopa challenges. L-dopa (LD; 6.25 mg/kg plus benserazide 15 mg/kg i.p.). Data are presented as medians and individual values (n=9-10) and groups are labelled according to their prior chronic treatment; **(a)** Total score, **(b)** Peak and **(c)** Duration of activity. Data were analysed by Kruskal-Wallis test. The data is a mean of the two final acute challenges.

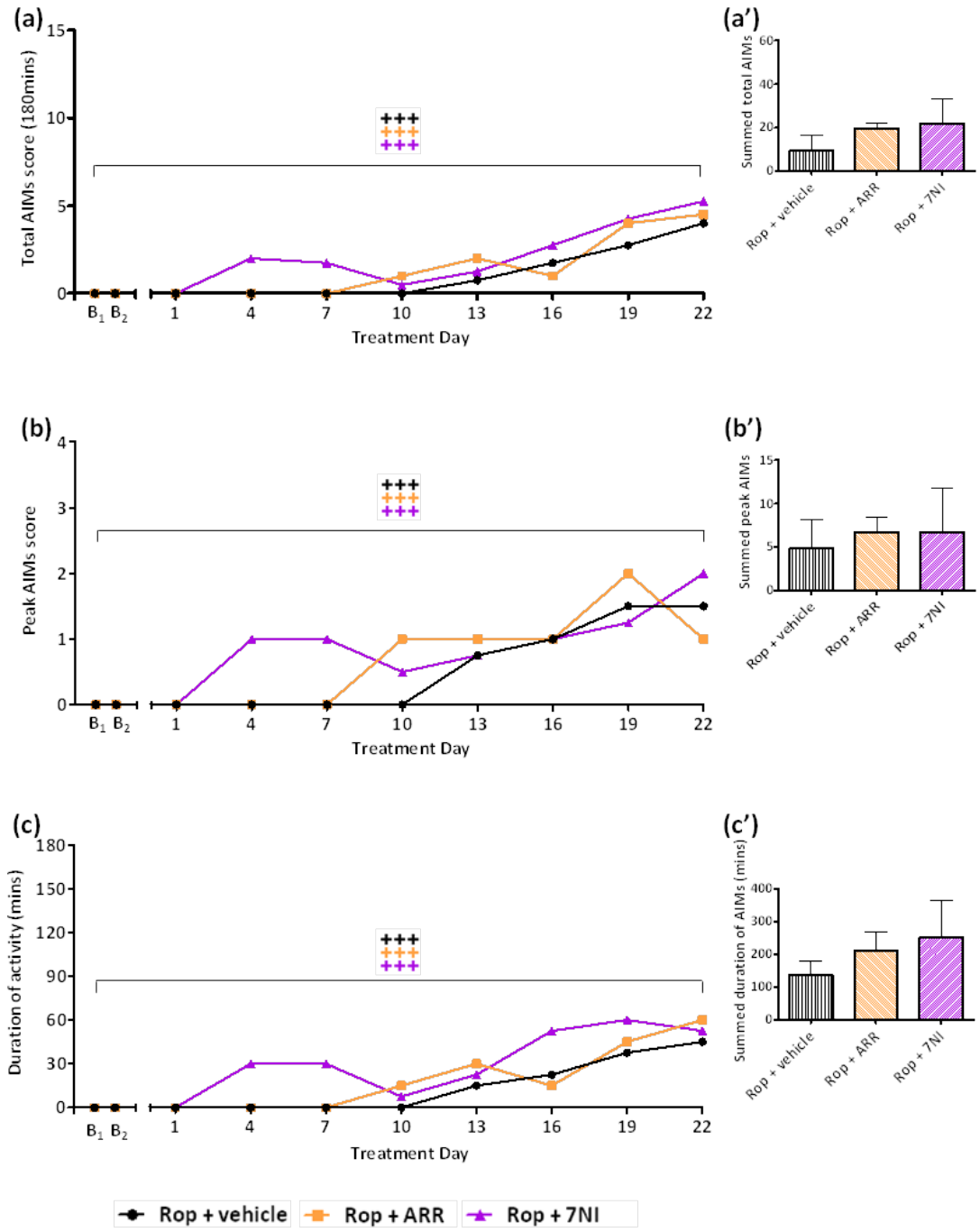

Figure 4-7 Locomotive AIMs following chronic treatment with ARR17477 or 7-NI plus ropinirole. ARR17477 (ARR; 1 mg/kg s.c), 7-NI (25 mg/kg i.p.) and ropinirole (Rop; 0.2 mg/kg i.p.) in 6-OHDA-lesioned rats. Data are all presented as medians and also a'-c' as interquartile ranges, (n=9-10); **(a)** Total score, **(b)** Peak score, **(c)** Duration of activity and **(a'-c')** Summed data over all treatment days for graphs a-c respectively. +++p<0.001 for time (colours used in (a-c) refer to key). Data (a-c) were analysed by 2-way-ANOVA and Friedman's test or (a'-c') by Kruskal-Wallis test (B_1:baseline 1 and B_2:baseline 2).

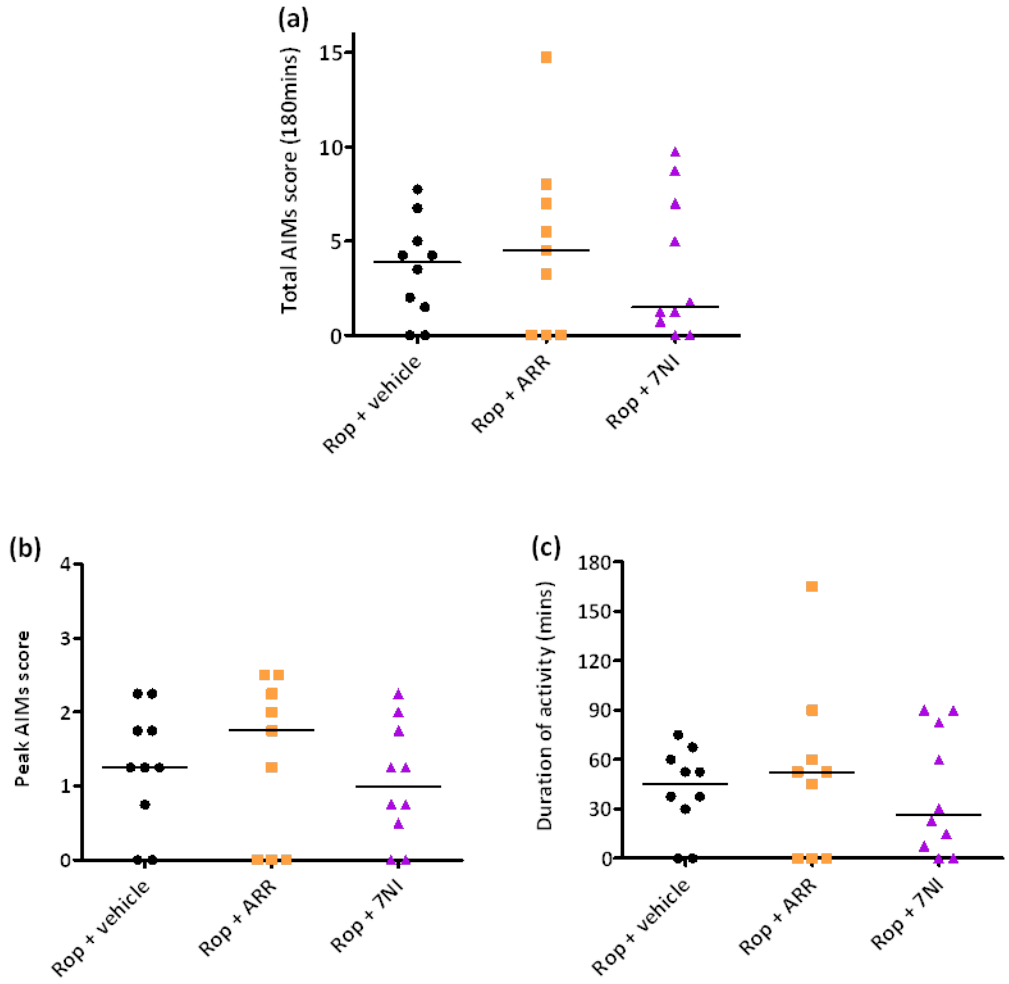

Figure 4-8 Locomotive AIMs following final ropinirole challenges. Ropinirole (Rop; 0.2 mg/kg i.p.). Data are presented as medians and individual values (n=9-10) and groups are labelled according to their prior chronic treatment; **(a)** Total score, **(b)** Peak and **(c)** Duration of activity. Data were analysed by Kruskal-Wallis test. The data is a mean of the two final acute challenges.

4.3.4 Axial, limb, orolingual and ALO AIMs in 6-OHDA-lesioned rats chronically treated with L-dopa plus nNOS inhibitor

4.3.4.1 Vehicle + L-dopa

L-dopa alone induced axial AIMs which significantly increased over the treatment period with total score reaching levels of 10 by day 7 and further escalating until reaching a maximum total of 24 on day 16 and then showing a small decrease thereafter (**Figure 4-9a**). On day 1 axial AIMs peaked at mild levels of 1 and then showed a similar pattern as total scores, reaching a maximal marked peak score of 3 on day 16 and continuing at moderate to marked levels until the final day of chronic treatment (**Figure 4-10a**). The median duration of axial AIMs increased from 55 min on day 1 to 120 min by day 13 and continued over a similar length of time up until day 22 (**Figure 4-11a**).

Limb AIMs also significantly increased over time growing from a total score of 9 on day 4 to 17 by day 7 and similar levels were maintained from here onwards and a maximal total score on 19 was observed on day 16 (**Figure 4-9b**). Peak limb AIMs increased from a mild-moderate score of 1.5 on day 4 to a moderate-marked 2.5 on day 7 which was also the maximal peak score and fluctuated between 2 and 2.5 over the remaining days of chronic treatment (**Figure 4-10b**). The duration of limb AIMs also increased from 90 min on day 4 to 130 min on day 7 and persisted for a comparable duration thereon (**Figure 4-11b**).

Orolingual AIMs showed a total score of 6 on day 1 and gradually increased reaching a maximum total of 18 on day 13 and continuing at similar levels until day 22 (**Figure 4-9c**), significantly escalating over time. Peak orolingual AIMs did not increase above median baseline levels until day 4 when a maximal peak score of 2 was exhibited and maintained until the final day of chronic treatment (**Figure 4-10c**). Following a similar trend as total scores the duration of orolingual AIMs increased from 8 min on day 1 to a maximum length of 100 min on day 13 and continued at similar mildly fluctuating levels thereafter (**Figure 4-11c**).

ALO AIMs reflected individual AIMs categories for L-dopa alone steadily increasing from a total score of 14 on day 1 reaching a maximum total score of 55 on day 16, and showing a small decline to 40 by day 22 (**Figure 4-9d**). Peak scores began mildly from above baseline on day 1 of L-dopa treatment showing moderate peak scores of 5 by day 7 and increasing to a maximum marked peak of 7 by day 16 and declining back to a similar peak of 6 by day 22 (**Figure 4-10d**). The duration of ALO AIMs increased from 60 min on day 1 to a maximum duration of 135 min by day 7 and persisted over a very similar time frame up until day 22 (**Figure 4-11d**).

4.3.4.2 nNOS inhibitor + L-dopa

7-NI significantly increased the duration of limb AIMs on day 4 compared to L-dopa alone and also the duration of ALO AIMs on days 4 and 22 of chronic treatment (**Figure 4-11b & d**). These findings were corroborated by the summed durations of activity which were significantly longer for L-dopa-induced

limb AIMs and ALO AIMs following 7-NI treatment (**Figure 4-11b' & d'**). There was some tendency for 7-NI to increase the total score and duration of axial AIMs, although there was no statistical effect (**Figure 4-9a-b & Figure 4-11a-b**).

There was no significant effect of ARR17477 on L-dopa-induced axial, limb, orolingual or ALO AIMs over the 22 day chronic treatment phase, as measured by totals, peaks or duration, compared to L-dopa alone (**Figure 4-9a-d, Figure 4-10a-d & Figure 4-11a-d**). There was also no significant effect of ARR17477 on summed total, peak or duration of axial, limb, orolingual or ALO AIMs (**Figure 4-9a'-d', Figure 4-10a'-d' & Figure 4-11a'-d'**).

4.3.4.3 L-dopa final acute challenges

Acute treatment with L-dopa alone induced a median total axial score of 17 (**Figure 4-12a**), with a moderate peak score of 2 (**Figure 4-12a'**) and duration of activity of 65 min (**Figure 4-12a''**) in animals which had been chronically treated with L-dopa alone.

Limb AIMs showed a total score of 14 (**Figure 4-12b**), a moderate peak score of 2 (**Figure 4-12b'**) and were expressed for 120 min (**Figure 4-12b''**).

Orolingual AIMs showed a total score of 9 (**Figure 4-12c**), a moderate peak score just below 2 (**Figure 4-12c'**) and were expressed for 45 min (**Figure 4-12c''**).

ALO AIMs showed a total score of 35 (**Figure 4-12d**), a moderate peak score of 6 (**Figure 4-12d'**) and were expressed for 125 min (**Figure 4-12d''**).

There was no significant effect of ARR17477 or 7-NI plus L-dopa chronic treatment on subsequent L-dopa-induced axial, limb, orolingual or ALO AIMs in terms of total scores, peaks or durations of activity (**Figure 4-12a-d, a'-d' & a''-d''**). However ARR17477 tended to reduce axial AIMs as measured by total scores (**Figure 4-12a**).

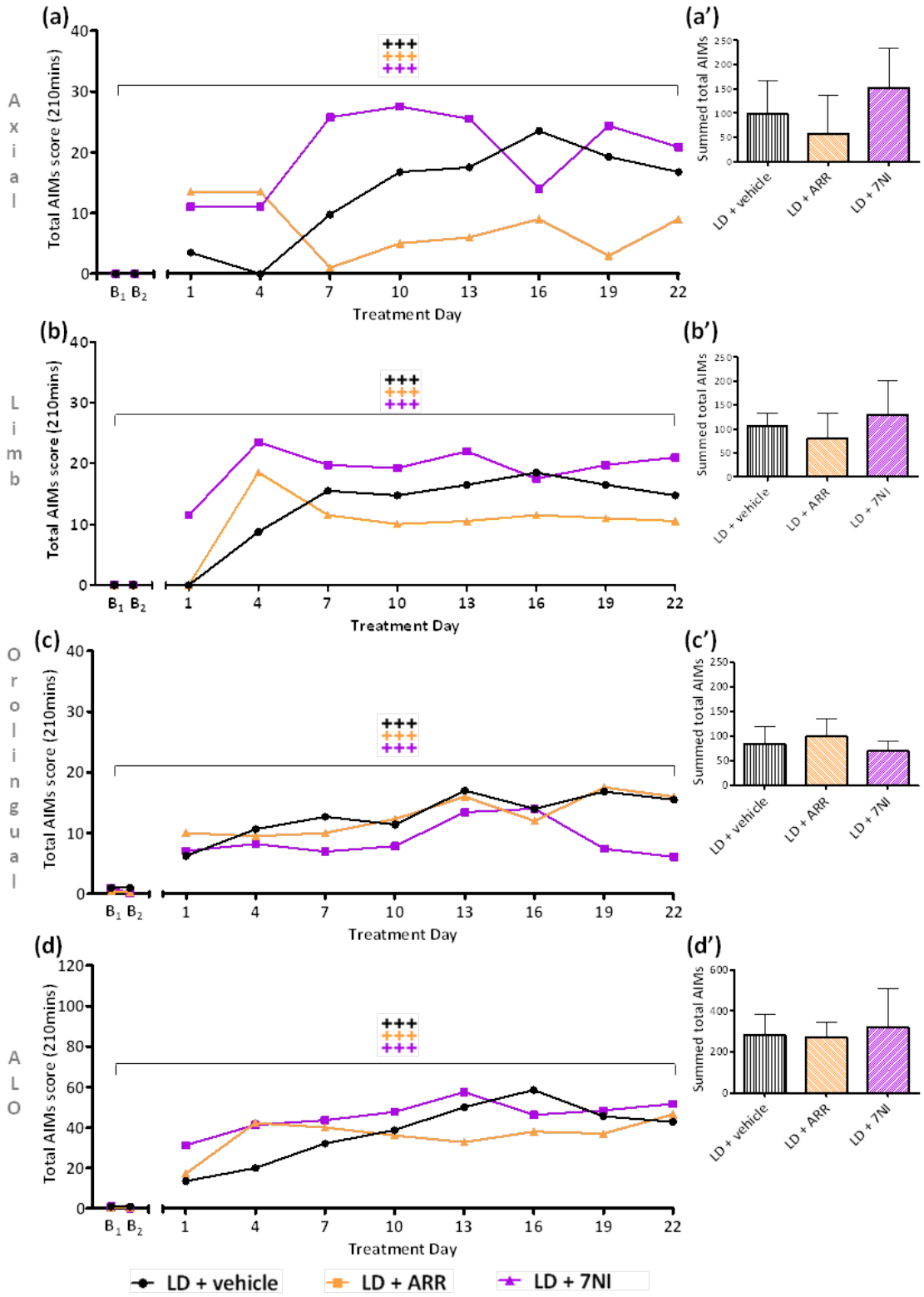

Figure 4-9 Total and Summed total scores for axial (a-a'), limb (b-b'), orolingual (c-c') and ALO AIMs (d-d') following chronic treatment with ARR17477 or 7-NI plus L-dopa. ARR17477 (ARR; 3, 6 or 12 mg/kg s.c), 7-NI (25 mg/kg i.p.) and L-dopa (LD; 6.25 mg/kg plus benserazide 15 mg/kg i.p.) treatment in 6-OHDA-lesioned rats. Data are all presented as medians and also a'-d' as interquartile ranges, (n=9-10); **(a-d)** Total scores and **(a'-d')** Summed data over all treatment days. +++p<0.001 for time (colours used in (a-d) refer to key). Data (a-d) were analysed by 2-way-ANOVA and Friedman's test or (a'-d') by Kruskal-Wallis test (B_1:baseline 1 and B_2:baseline 2).

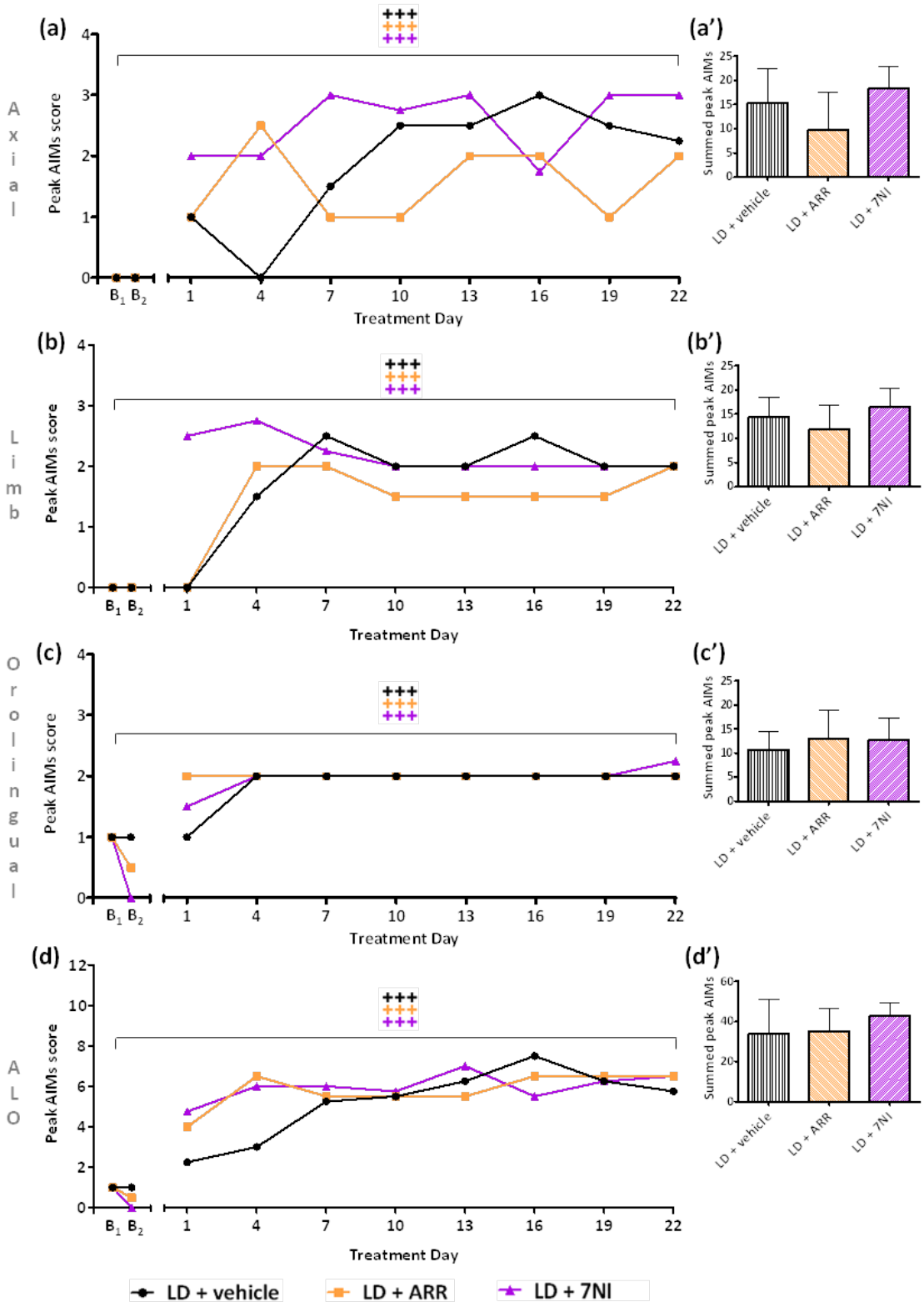

Figure 4-10 Peak and Summed peak scores for axial (a-a'), limb (b-b'), orolingual (c-c') and ALO AIMs (d-d') following chronic treatment with ARR17477 or 7-NI plus L-dopa. ARR17477 (ARR; 3, 6 or 12 mg/kg s.c), 7-NI (25 mg/kg i.p.) and L-dopa (LD; 6.25 mg/kg plus benserazide 15 mg/kg i.p.) treatment in 6-OHDA-lesioned rats. Data are all presented as medians and also a'-d' as interquartile ranges, (n=9-10); **(a-d)** Total scores and **(a'-d')** Summed data over all treatment days. +++p<0.001 for time (colours used in (a-d) refer to key). Data (a-d) were analysed by 2-way-ANOVA and Friedman's test or (a'-d') by Kruskal-Wallis test (B_1:baseline 1 and B_2:baseline 2).

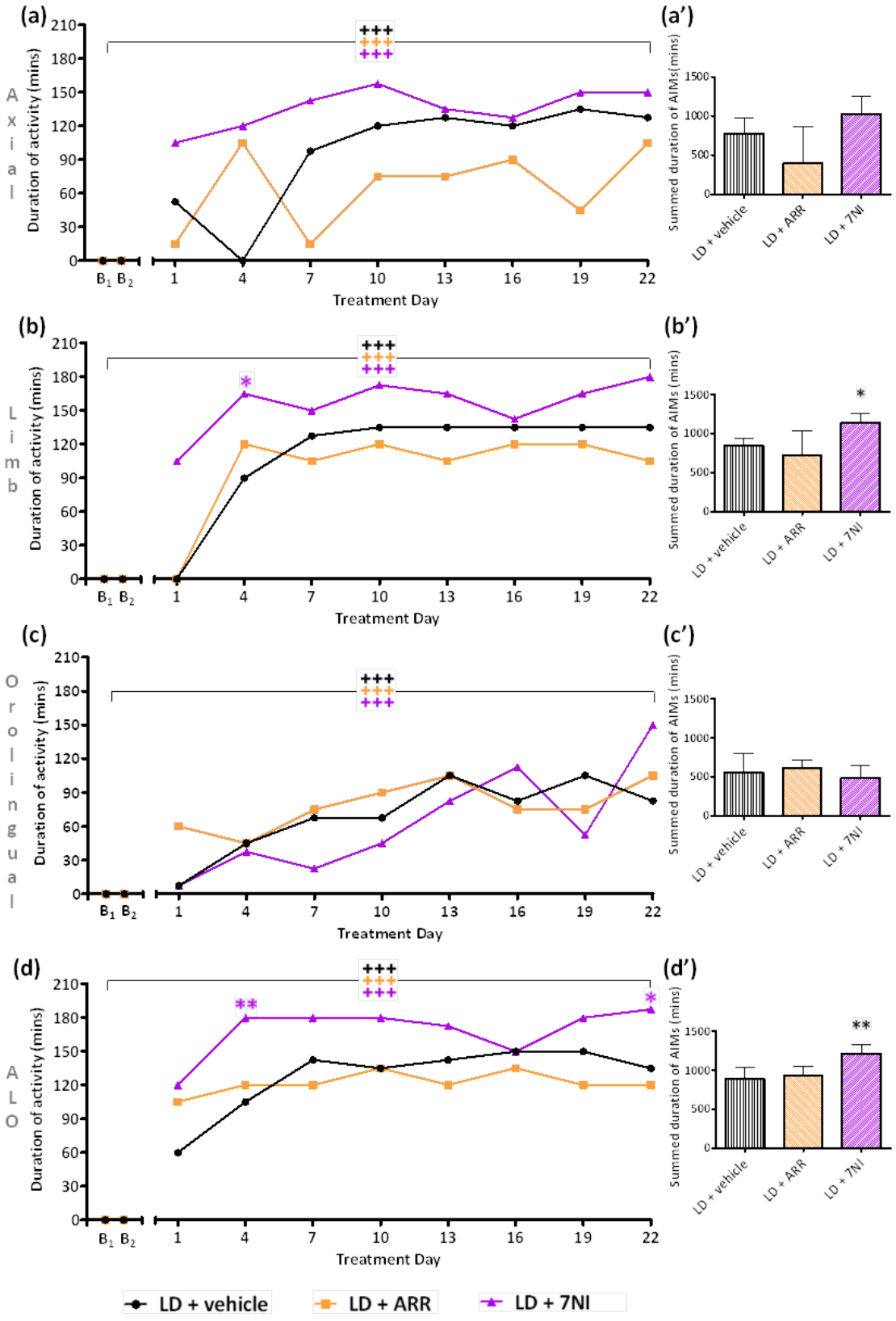

Figure 4-11 Duration and Summed duration of activity scores for axial (a-a'), limb (b-b'), orolingual (c-c') and ALO AIMs (d-d') following chronic treatment with ARR17477 or 7-NI plus L-dopa. ARR17477 (ARR; 3, 6 or 12 mg/kg s.c), 7-NI (25 mg/kg i.p.) and L-dopa (LD; 6.25 mg/kg plus benserazide 15 mg/kg i.p.) treatment in 6-OHDA-lesioned rats. Data are all presented as medians and also a'-d' as interquartile ranges, (n=9-10); **(a-d)** Total scores and **(a'-d')** Summed data over all treatment days. +++p<0.001 for time; *p<0.05 **p<0.01 for treatment compared to rop + vehicle (colours used in (a-d) refer to key). Data (a-d) were analysed by 2-way-ANOVA and Friedman's test, or Kruskal-Wallis test followed by Dunn's, or (a'-d') by Kruskal-Wallis test followed by Dunn's (B_1:baseline 1 and B_2:baseline 2).

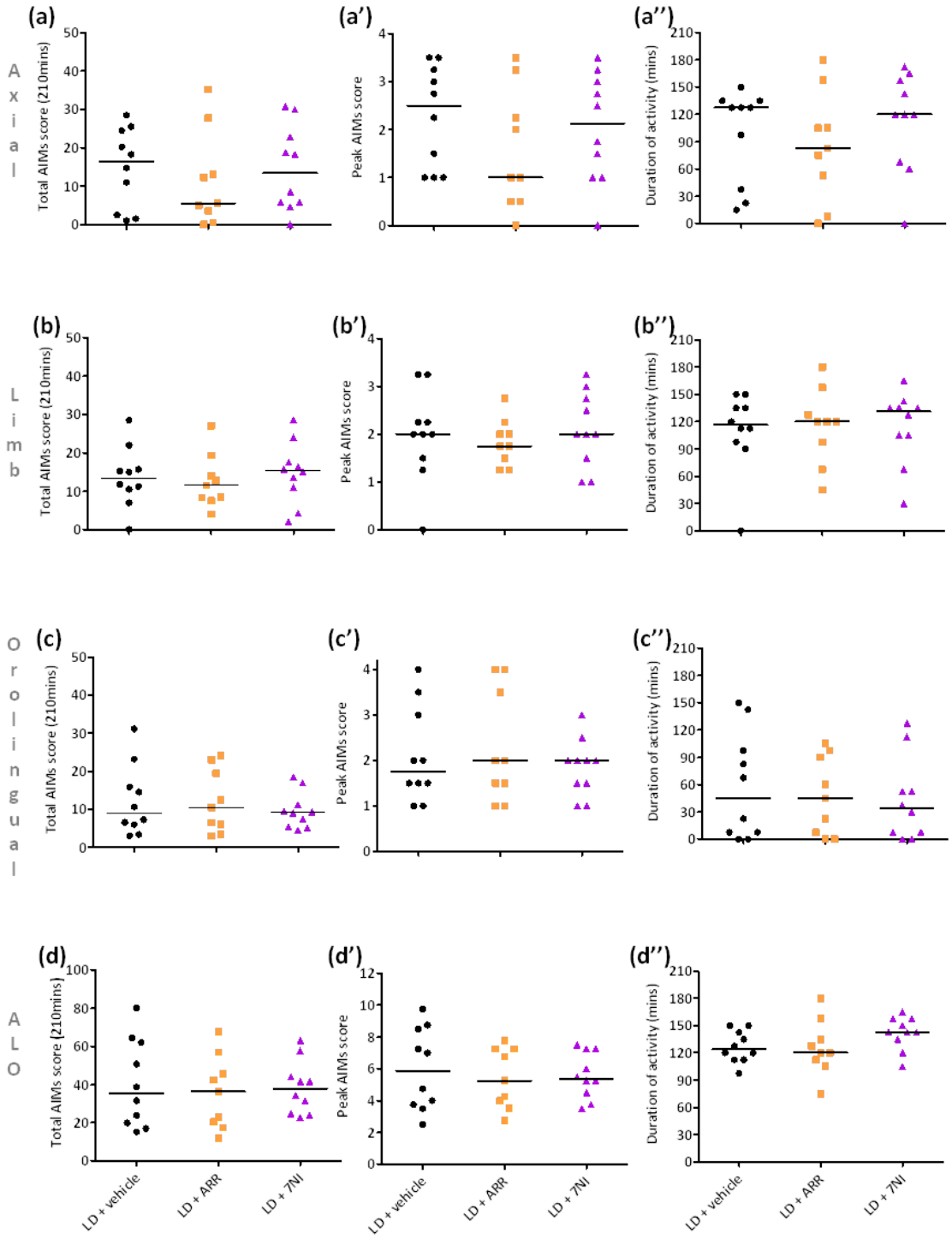

Figure 4-12 Final L-dopa challenge data for axial (a-a''), limb (b-b''), orolingual (c-c'') and ALO AIMs (d-d''). L-dopa (LD; 6.25 mg/kg plus benserazide 15 mg/kg i.p.). Data are presented as medians and individual values (n=9-10) and groups are labelled according to their prior chronic treatment; **(a-d)** Total score, **(a'-d')** Peak score and **(a''-d'')** Duration of activity. Data were analysed by Kruskal-Wallis test. The data is a mean of the two final acute challenges.

4.3.5 Axial, limb, orolingual and ALO AIMs in 6-OHDA-lesioned rats chronically treated with ropinirole plus nNOS inhibitor

4.3.5.1 Vehicle + ropinirole

Ropinirole alone induced axial AIMs which significantly increased over the chronic treatment period resulting in a total score of 3 on day 7 very gradually increasing to 5 by day 13 and 7.5 by day 22 (**Figure 4-13a**). On day 7 axial AIMs had a moderate peak score of 2, which was also the maximum peak score seen over the 22 day of chronic treatment (**Figure 4-14a**). By day 10 animals showed a reduced peak axial score of just above 1 and these mild AIMs gradually increased back up to 2 by day 22. The duration of axial AIMs was 30 min on day 7, extending to 60 min by day 10 with minor fluctuations from here on and finally showing axial activity for 70 min on day 22 (**Figure 4-15a**).

Limb AIMs significantly increased over the chronic treatment period where a total score of 1 was recorded on day 16, decreasing to give a median total score of 0.5 on all subsequent assessment days (**Figure 4-13b**). The peak limb AIMs score for day 16-22 was a very mild 0.5 (**Figure 4-14b**), whilst the duration of activity was 15 min on day 16 shortening to just under 10 min on the remaining days (**Figure 4-15b**).

Orolingual AIMs scored a total of 2 on day 10 and showed a gradual increase over time reaching 5.5 on day 22 (**Figure 4-13c**), significantly rising over the full treatment period. Peak orolingual AIMs scored 1 at baseline and did not rise beyond this level until day 19 when the score was a mild-moderate 1.75, being displayed similarly on day 22 (**Figure 4-14c**). The duration of orolingual AIMs activity was 15 min on day 19 increasing to 25 min on day 22 (note only periods with scores above 1 are used to calculate the duration of orolingual AIMs – see section 2.3.2.2) (**Figure 4-15c**).

ALO AIMs showed a total score of only 2.5 on day 4, accounting for the orolingual AIMs observed early on, and gradually increased in keeping with the individual AIMs subtypes reaching a final total score of 13 on day 22 (**Figure 4-13d**). Peak ALO AIMs of a mild score of 2 were seen from day 7 onwards increasing to more moderate levels of 4-4.5 over days 19-22 (**Figure 4-14d**). The duration of ALO AIMs was 30 min on day 7, increasing to 45 min by day 13 and reaching 60 min from day 19 onwards (**Figure 4-15d**).

4.3.5.2 nNOS inhibitor + ropinirole

7-NI in combination with ropinirole induced axial AIMs on day 4 that were significantly higher scoring than ropinirole alone as measured by totals and peak scores (**Figure 4-13a & Figure 4-14a**), and also significantly longer lasting as measured by duration of activity (**Figure 4-15a**). Additionally on day 16, 7-NI significantly increased the duration of activity of axial AIMs as compared to ropinirole treatment alone (**Figure 4-15a**). The display of axial AIMs was effectively extended by 7-NI treatment, first appearing on day 4 (with a total score of 7, peak score of 2 and duration of activity of 60 min compared to 0 for ropinirole alone treatment on the same day) rather than day 7 and continuing for 90 min rather than 50 min on day 16. Meanwhile there was no statistically significant effect of 7-NI treatment on limb,

orolingual or ALO AIMs as measured by totals, peaks or duration throughout the 22 days of treatment (**Figure 4-13b-d, Figure 4-14b-d & Figure 4-15b-d**). Summed total scores over the chronic treatment phase also showed 7-NI significantly increased the duration of ropinirole-induced axial AIMs (**Figure 4-15a'**), although these data did not reveal any other significant effects (**Figure 4-13a'-d', Figure 4-14a'-d' & Figure 4-15b'-d'**). There was however a tendency for 7-NI to increase summed total axial AIMs in addition to summed total, peak and duration of limb AIMs (**Figure 4-13b' and d' & Figure 4-15b'**).

ARR17477 plus ropinirole had no significant effect on axial, limb, orolingual or ALO AIMs activity over the chronic treatment period as measured by totals, peaks or duration, compared to ropinirole alone (**Figure 4-13a-d, Figure 4-14a-d & Figure 4-15a-d**). There was also no significant effect of ARR17477 on summed total, peak or duration of axial, limb, orolingual or ALO AIMs (**Figure 4-13a'-d', Figure 4-14a'-d' & Figure 4-15b'-d'**)

4.3.5.3 Ropinirole final acute challenges

Acute treatment with ropinirole alone induced axial AIMs totalling 8 (**Figure 4-16a**) with a moderate peak score of 2 (**Figure 4-16a'**) and duration of activity lasting 65 min (**Figure 4-16a''**), in animals which had been chronically treated with ropinirole alone.

Limb AIMs showed a total score of 1.5 (**Figure 4-16b**), a mild peak score of 1 (**Figure 4-16b'**) and were expressed for a period of 15 min (**Figure 4-16b''**).

Orolingual AIMs showed a total score of 2.5 (**Figure 4-16c**), a mild peak score just above 1 (**Figure 4-16c'**) and were expressed for 10 min (**Figure 4-16c''**).

ALO AIMs showed a total score of 12 (**Figure 4-16d**), a moderate peak score of 4 (**Figure 4-16d'**) and were expressed for 60 min (**Figure 4-16d''**).

There was no significant effect of ARR17477 or 7-NI plus ropinirole chronic treatment on subsequent ropinirole induced axial, limb, orolingual or ALO AIMs in terms of total scores, peaks or durations of activity (**Figure 4-16**a-**d, Figure 4-16a'-d' & Figure 4-16a''-d''**). However ARR17477 tended to increase the duration of activity of orolingual AIMs (**Figure 4-16c''**), and 7-NI tended to increase the total and duration of limb AIMs (**Figure 4-16b & b''**).

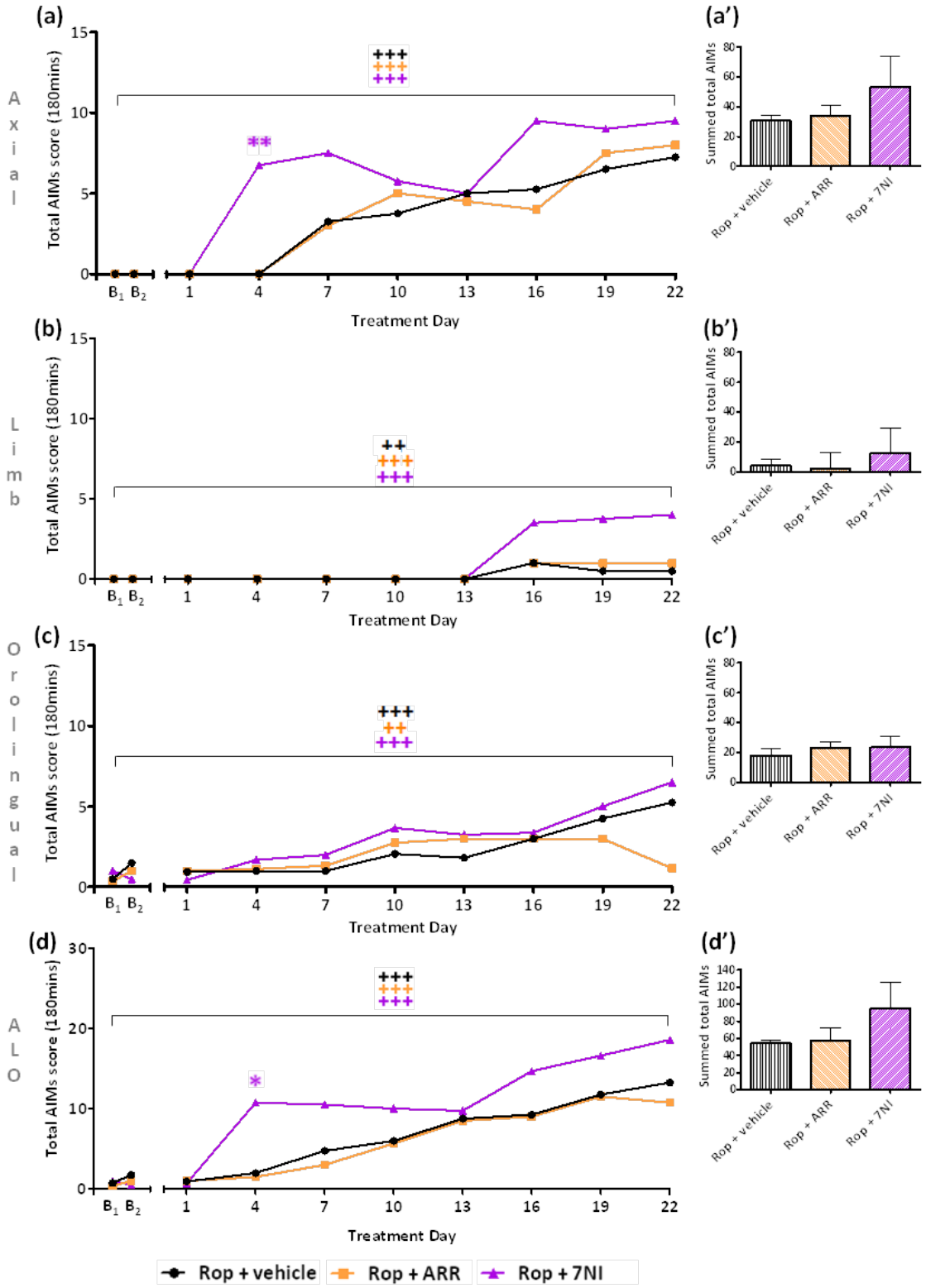

Figure 4-13 Total and Summed total scores for axial (a-a'), limb (b-b'), orolingual (c-c') and ALO AIMs (d-d') following chronic treatment with ARR17477 or 7-NI plus ropinirole. ARR17477 (ARR; 3, 6 or 12 mg/kg s.c), 7-NI (25 mg/kg i.p.) and (Rop; 0.2 mg/kg i.p.) treatment in 6-OHDA-lesioned rats. Data are all presented as medians and also a'-d' as interquartile ranges, (n=9-10); (a-d) Total scores and (a'-d') Summed data over all treatment days. ++p<0.01, +++p<0.001 for time; *p<0.05 **p<0.01 for treatment compared to rop + vehicle (colours used in (a-d) refer to key). Data (a-d) were analysed by 2-way-ANOVA and Friedman's test, or Kruskal-Wallis test followed by Dunn's, or (a'-d') by Kruskal-Wallis test (B_1 :baseline 1 and B_2 :baseline 2).

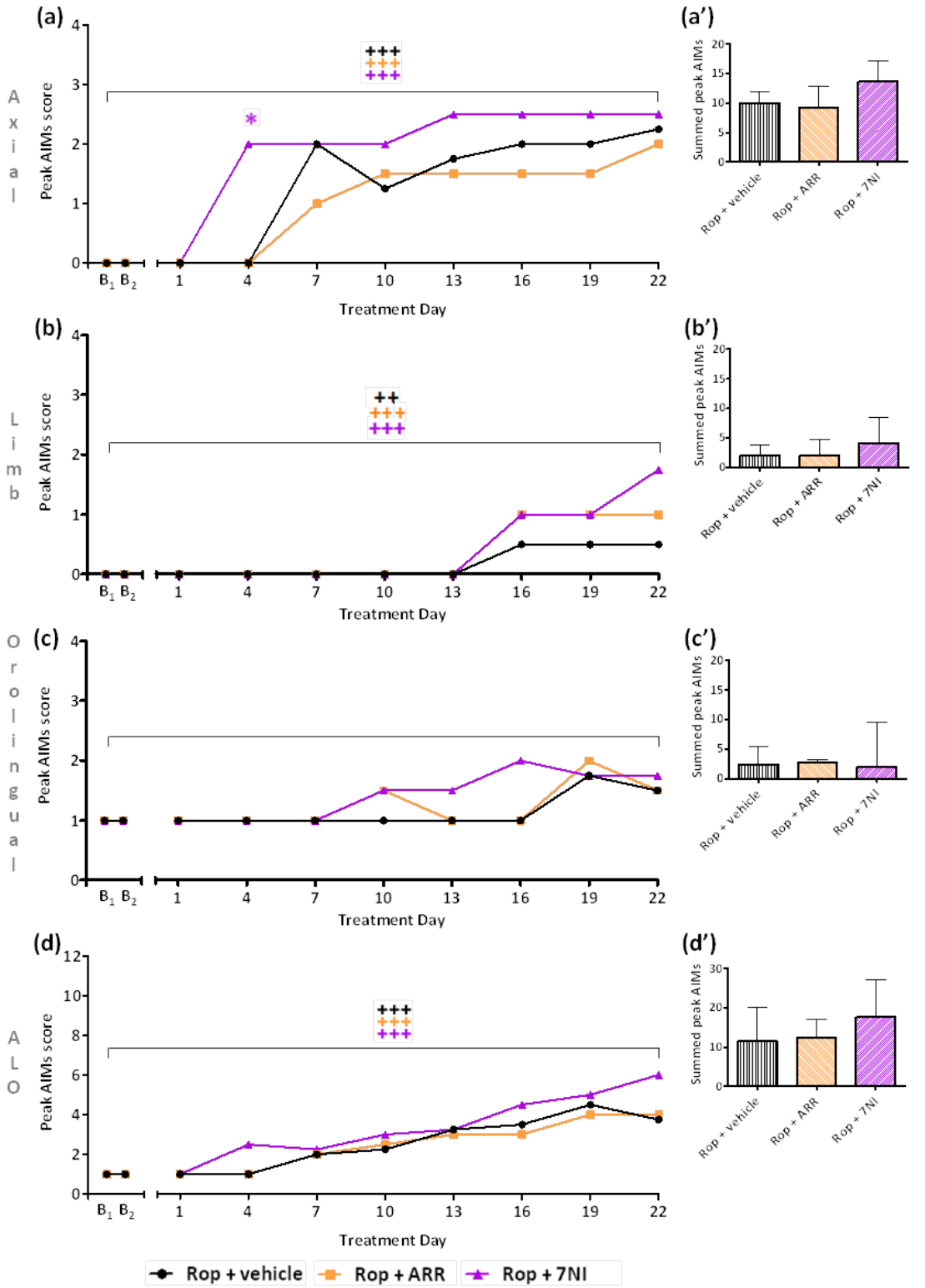

Figure 4-14 Peak and Summed peak scores for axial (a-a'), limb (b-b'), orolingual (c-c') and ALO AIMs (d-d') following chronic treatment with ARR17477 or 7-NI plus ropinirole. ARR17477 (ARR; 3, 6 or 12 mg/kg s.c), 7-NI (25 mg/kg i.p.) and (Rop; 0.2 mg/kg i.p.) treatment in 6-OHDA-lesioned rats. Data are all presented as medians and also a'-d' as interquartile ranges, (n=9-10); **(a-d)** Total scores and **(a'-d')** Summed data over all treatment days. ++p<0.01, +++p<0.001 for time; *p<0.05 for treatment compared to rop + vehicle (colours used in (a-d) refer to key). Data (a-d) were analysed by 2-way-ANOVA and Friedman's test, or Kruskal-Wallis test followed by Dunn's, or (a'-d') by Kruskal-Wallis test (B_1 :baseline 1 and B_2 :baseline 2).

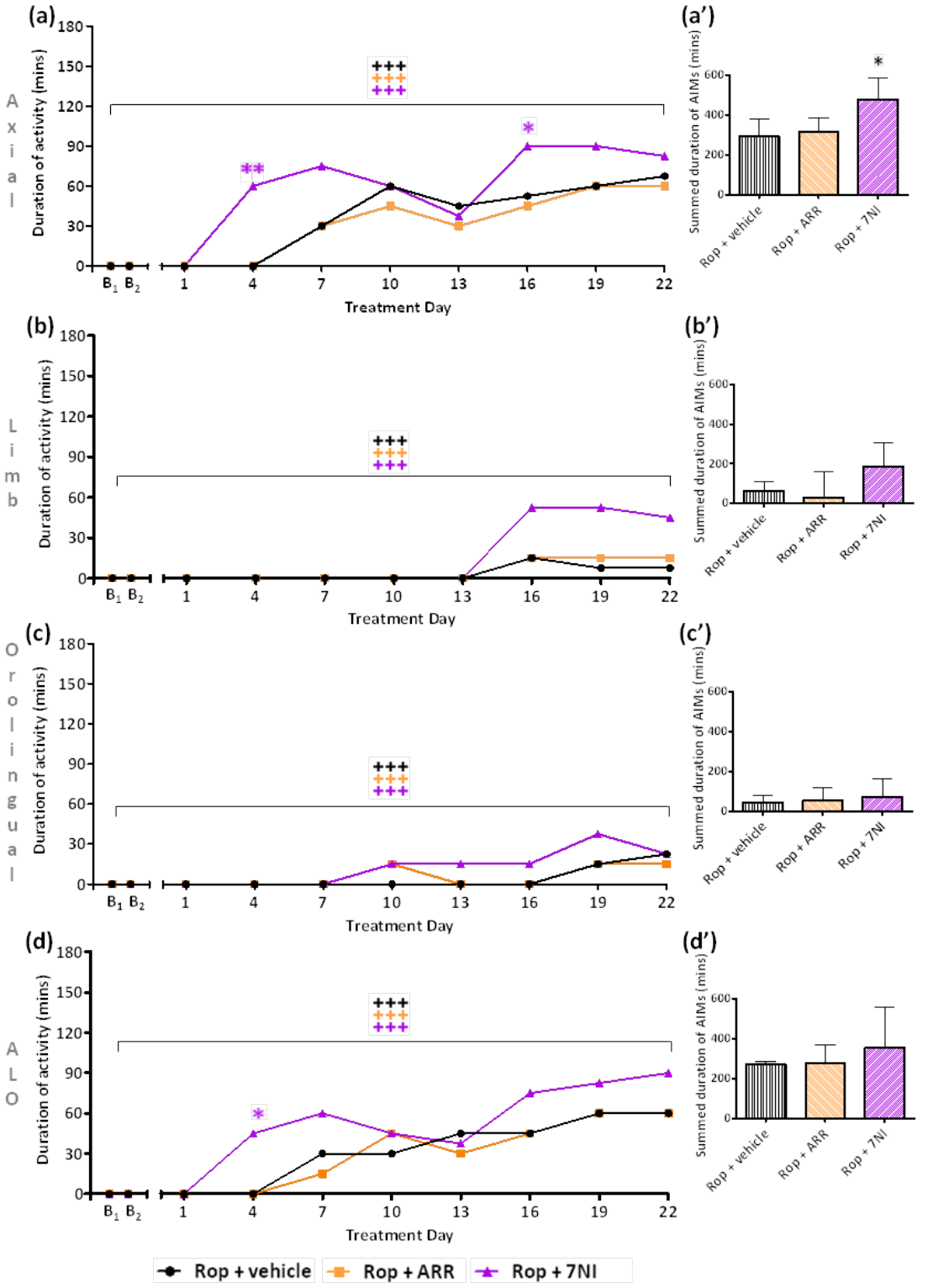

Figure 4-15 Duration and Summed duration of activity scores for axial (a-a'), limb (b-b'), orolingual (c-c') and ALO AIMs (d-d') following chronic treatment with ARR17477 or 7-NI plus ropinirole. ARR17477 (ARR; 3, 6 or 12 mg/kg s.c), 7-NI (25 mg/kg i.p.) and (Rop; 0.2 mg/kg i.p.) treatment in 6-OHDA-lesioned rats. Data are all presented as medians and also a'-d' as interquartile ranges, (n=9-10); (a-d) Total scores and (a'-d') Summed data over all treatment days. +++p<0.001 for time; *p<0.05 **p<0.01 for treatment compared to rop + vehicle (colours used in (a-d) refer to key). Data (a-d) were analysed by 2-way-ANOVA and Friedman's test, or Kruskal-Wallis test followed by Dunn's, or (a'-d') by Kruskal-Wallis test followed by Dunn's (B_1:baseline 1 and B_2:baseline 2).

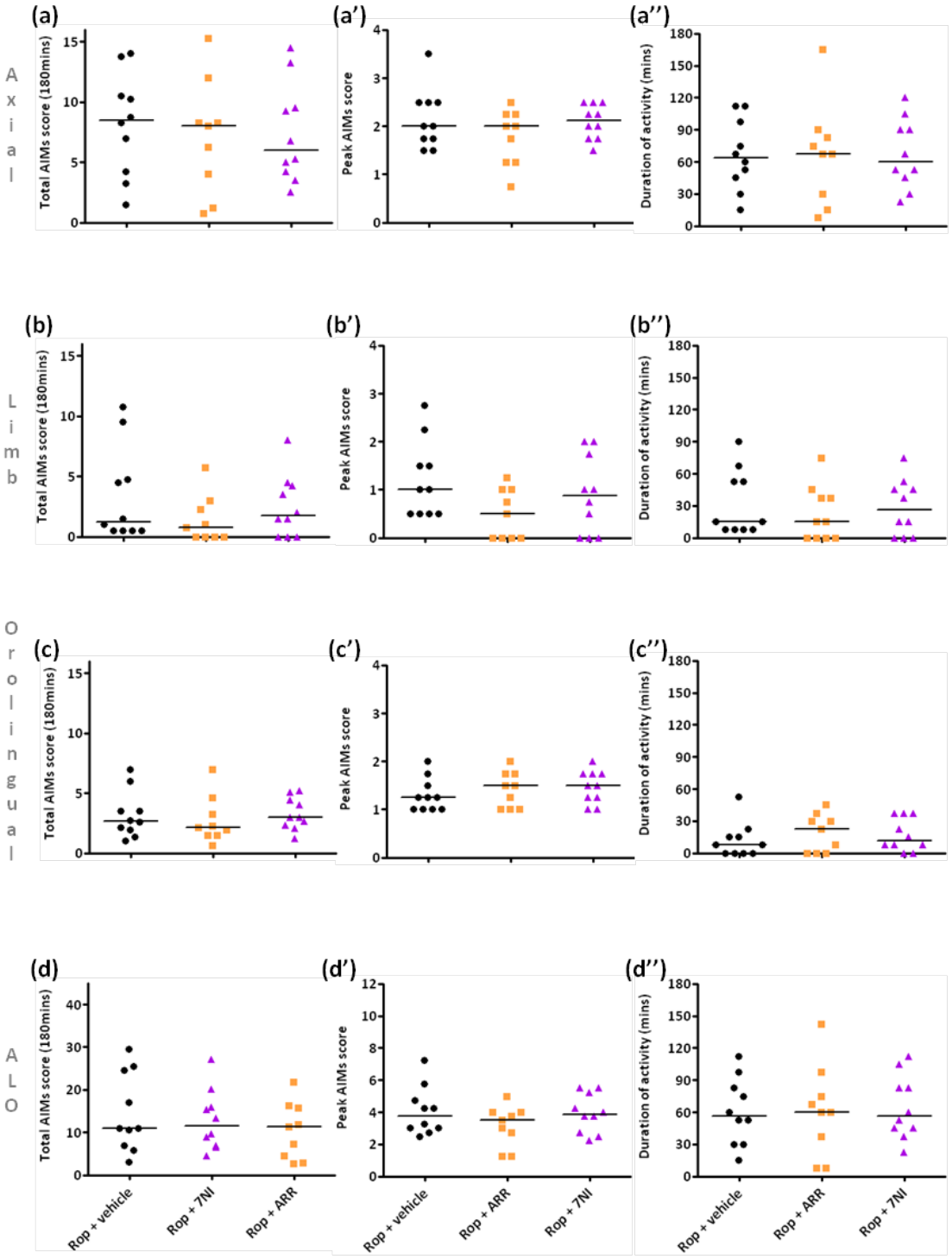

Figure 4-16 Final ropinirole challenge data for axial (a-a''), limb (b-b''), orolingual (c-c'') and ALO AIMs (d-d''). Ropinirole (Rop; 0.2 mg/kg i.p.) treatment. Data are presented as medians and individual values (n=9-10) and groups are labelled according to their prior chronic treatment; **(a-d)** Total score, **(a'-d')** Peak score and **(a''-d'')** Duration of activity. Data were analysed by Kruskal-Wallis test. The data is a mean of the two final acute challenges.

4.4 Discussion

These studies investigated whether the administration of nNOS inhibitors with early-symptomatic treatment of PD could potentially avoid the emergence of dyskinesia. It was hypothesised that the selective inhibition of neuronal NOS would prevent the induction of dyskinesia by L-dopa, or the dopamine agonist ropinirole, and these effects were tested in the drug naive 6-ODHDA-lesioned rat.

Initial studies set out to establish the dose of the long-acting and irreversible nNOS inhibitor ARR17477 appropriate for use on a chronic basis, owing to concerns of drug accumulation arising from daily treatment. The dose of the short-acting nNOS inhibitor 7-NI was selected based on *ex vivo* results from Chapter 3 supported by the literature. These doses were then taken forward to *in vivo* experiments to explore their effects on induction and development of AIMs from the first exposure of lesioned rats to dopaminergic treatment.

4.4.1 Inhibition of nNOS by chronic treatment with 7-NI

The dose of 7-NI (25 mg/kg i.p.) was chosen based on results from the *ex vivo* and acute study of effects of nNOS inhibitors on dyskinesia expression showing a significant reduction in nNOS activity in the cerebellum and striatum of 58 % and 41 % respectively (See Chapter 3, section 3.2.3). This dose was also consistent with published chronic studies of 7-NI in rodents; Bush and Pollack (2001) showed 7-NI 25 mg/kg i.p. every 2 h for 14 h lead to a sustained ~50 % decrease in hippocampal NO_2^- and NO_3^- as measured by microdialysis, whilst Schulz *et al.* (1995) and Przedborski *et al.* (1996) employed 7-NI 25 mg/kg s.c. dosed every 8 h for 2-4 days to achieve ongoing nNOS inhibition, although *ex vivo* data were not presented. Additionally Mackenzie *et al.* (1994) showed repeated administration was necessary every 4 hfor 20 h for significant inhibiton of nNOS (51-60 %) at 24 h demonstrating that repeated dosing of rats with 7-NI does not lead to a cumulative inhibition of nNOS ,being similar to the inhibition measured at 4 h. Due to this evidence combined with the reversible nature and short half life of 7-NI confirmed by other investigations (Moore *et al.*, 1993b), no adverse behavioural effects of chronic 7-NI treatment were anticipated using the once daily dosing regimen employed in the present study, or indeed observed. This outcome was in accordance with a study which employed 7-NI (25 mg/kg s.c.) daily treatment for 4 weeks in naïve rats without seeing any unexpected effects (Wangensteen *et al.*, 2006).

4.4.2 Inhibition of nNOS by chronic treatment with ARR17477

Initial studies showed that ARR17477 inhibited nNOS in a dose-related manner, with significant effects between 1 and 6 mg/kg (s.c.) at 12 h after four consecutive days of treatment. Following this study a dose of 1 mg/kg (s.c) was further investigated after the same dosing regimen to confirm nNOS inhibition was significant at a 1 h time point, where plasma levels of dopaminergic treatment to be used for *in vivo* studies would be expected to peak. After four days of treatment, ARR17477 (1 mg/kg s.c.) inhibited nNOS reducing its activity by 95 % at one hour following administration. Previous studies have shown ARR17477 to significantly reduce nNOS activity after an acute dose of 10 mg/kg i.v. (Reif *et al.*, 2000),

but this thesis represents the first time that the effects of chronic administration of ARR17477 on nNOS activity have been reported. Inhibition of nNOS following this repeated scheme of administration produced levels of inhibition almost twice the extent found following a single dose of ARR17477 at the highest dose (12 mg/kg s.c.) in comparable tissue, as measured in Chapter 3. Importantly after dosing with ARR17477 (1 mg/kg s.c.) nNOS activity was reduced by 95 % at peak effect of L-dopa and dopamine agonist activity, and clearly remained significantly decreased for the major duration of dopaminergic activity according to the persisting nNOS inhibition measured at a 12 h time-point. The cumulative effect seen following chronic treatment further supports the long-lasting and irreversible nature of NOS inhibition by ARR17477. As no side-effects were apparent in animals following the 4 days of treatment and high levels of nNOS inhibition were conferred by this dose (1 mg/kg s.c.) of ARR17477 it was considered appropriate to ensure chronic nNOS inhibition throughout the study.

4.4.3 The effect of chronic nNOS inhibition on L-dopa-induced AIMS

As expected, L-dopa caused a significant increase in the expression of all AIMS sub-types over the treatment period. The same was also true for L-dopa administered in combination with the nNOS inhibitors ARR17477 and 7-NI. Additionally chronic ARR17477 or 7-NI treatment alone was found to have no effect on AIMs suggesting that there may be no dopamine associated changes induced by either nNOS inhibitor in the absence of L-dopa or ropinirole, or indeed if there are they appear insufficient to result in AIMs.

Although there was no significant reduction of L-dopa-induced AIMs by either nNOS inhibitor during chronic treatment, summed totals showed 7-NI significantly increased the duration of limb and ALO AIMs compared to vehicle treatment. Additionally 7-NI tended to increase axial AIMs but these effects did not reach statistical significance. Meanwhile there was a trend for ARR17477 to reduce axial AIMs as measured by total scores in final L-dopa only challenges, and locomotive AIMS as measured by summed scores and final L-dopa only challenges.

As discussed in Chapter 3, where 7-NI also caused an increase in certain AIMs categories, this may be due to MAOB inhibitor activity (Castagnoli *et al.*, 1997; Di Monte *et al.*, 1997; Desvignes *et al.*, 1999) prolonging the presence of extracellular dopamine and also therefore the resulting dyskinesia (Lamensdorf *et al.*, 1996; Kadieva & Mutsueva, 2005). Notably the resulting prolongation of antiparkinsonian action afforded by MAOB adjunct therapy in the clinic usually permits the levodopa dose to be reduced and hence the level of associated dyskinesia falls, but in this case where L-dopa dose is unchanged the opposite effect is likely (Fernandez et al., 2007). Additionally possible eNOS effects of 7-NI could impact on dyskinesia as a consequence of changes in vascular tone (Zagvazdin *et al.*, 1996; Ayajiki *et al.*, 2001), although this seems a less likely explanation as a sub-maximal dose of 7-NI was used in the present chronic studies.

The tendency of ARR17477 to show some reduction in L-dopa-induced dyskinesia suggests that nNOS inhibition could be driving a decrease in AIMs but this may be insufficient and/or may be competing

against other properties of the compound acting to oppose this effect. ARR1477 shows significant binding affinity for the vesicular monoamine transporter (VMAT), the norepinephrine transporter (NET), the dopamine transporter (DAT) and adrenergic α2 receptors (see Appendix, Table 0-1). Inhibition of VMAT which controls vesicular storage of the monoamines including dopamine would likely exacerbate the fluctuations in extracellular dopamine levels associated with dyskinesia as would NET inhibition as this also clears excess dopamine in the denervated striatum (Lee *et al.*, 2006; Arai *et al.*, 2008). Inhibition of DAT has been associated with increased dyskinesia (Nutt *et al.*, 2004) although a reduction in dyskinesia has also been reported (Pearce *et al.*, 2002). Additionally antagonist activity at adrenergic α2 receptors has shown antidyskinetic effects pre-clinically (although notably clinical trials have been unsupportive) suggesting that α2 stimulation may promote dyskinesia (Fox *et al.*, 2001; Savola *et al.*, 2003). ARR17477 may therefore have properties which can contribute to worsen dyskinesia in addition to its affinity to nNOS. This characteristic was supported by the final acute challenges with L-dopa alone, for axial and locomotive AIMs in the present study, showing a trend for a reduction in scores/duration, although this was not reflected by other AIMs subcategories. This discrepancy may be due to axial and locomotive AIMs being more resilient to further L-dopa exposure than the other forms as it is proposed that different anatomical brain regions underlie the varied AIMs (Winkler *et al.*, 2002). For example studies concentrating on especially localised dopamine depletion have shown the ventrolateral caudate-putamen is associated with forelimb control whilst medial caudate-putamen, is associated with development of rotations thus relating to limb and locomotive AIMs respectively, whilst dopaminergic receptor changes in the subthalamic nucleus are associated with orofacial dyskinesia (Salamone *et al.*, 1993; Mehta *et al.*, 2000). Further evidence would be required to verify these differences, although changes in activity of the direct and indirect striatal pathways are considered to be predominantly involved with either choreic or dystonic sub-types of dyskinesia respectively (Bezard *et al.*, 2001; Schuster *et al.*, 2008).

Lack of significant efficacy may be due to non-nNOS-specific effects of the inhibitors used in these studies. Currently, ARR17477 is the most potent and selective nNOS specific inhibitor described in the literature which can be administered systemically, as many more selective inhibitors are let down by an inability to penetrate the blood-brain barrier (Lawton *et al.*, 2009). ARR17477 is also known to be more potent and nNOS-selective than 7-NI with an IC_{50} of 0.07 µM compared to 8.3 µM respectively (Vallance & Leiper, 2002), and these factors may explain its relatively more beneficial effects on AIMS in the rat model.

Presented here is the first study to investigate the effect of chronic selective inhibition of nNOS in L-dopa priming for AIMs/dyskinesia. Recently Padovan-Neto *et al.* (2011) showed the non-selective eNOS and nNOS inhibitor, NG-nitro-L-Arginine (L-NOARG), reduced limb and orolingual AIMs at 60 min after first L-dopa exposure and also reduced locomotive AIMS at 120 min in 6-OHDA-lesioned rats. However no subsequent days of L-dopa priming were considered in this case. Again animals were only assessed at 60 and 120 min following L-dopa treatment and the dose of L-dopa administered was astonishingly 16x greater than used in the experiments presented here and indeed commonly employed in AIMs

experiments (e.g.Taylor *et al.*, 2005; Dekundy *et al.*, 2007; Monville *et al.*, 2009). These differences suggest that in fact eNOS inhibition in addition to nNOS may indeed be advantageous in reducing AIMs specifically when a supra-therapeutic dose of L-dopa is employed. However the effect of such a high dose of L-dopa needs to be considered, and suggests a non-clinically relevant finding.

There are a range of studies confirming that AIMs can indeed be reduced during the initial process of chronic L-dopa exposure in the 6-OHDA-lesioned rat model. There is considerable evidence for an important role of glutamate in AIMs priming. Most recently the metabotropic glutamate receptor type 5 (mGluR5) antagonist Fenobam was shown to reduce the development of AIMs, measured at peak dopaminergic effect, when administered in combination with L-dopa from first exposure (Rylander *et al.*, 2010). Importantly, this reduction in AIMS was not due to motor impairment effects of the drug. L-dopa was used at a comparable dose (6 mg/kg) to that applied in the studies presented here and administered on a once daily basis resulting in a significant decrease in ALO AIMs during chronic treatment. Similarly the mGluR5 antagonist MTEP effectively inhibited AIMs development induced by L-dopa (Mela *et al.*, 2007; Rylander *et al.*, 2009). The AMPA receptor antagonist IEM 1460 also reduced L-dopa induced priming for ALO AIMs in drug naïve 6-OHDA-lesioned rats suggesting a key involvement of AMPA receptors in the priming process (Kobylecki *et al.*, 2010).

Manipulation of additional neurotransmitter pathways in 6-OHDA-lesioned rats has also shown promise in treatment for dyskinesia. Chronic administration of a combination of 5-HT1A and 5-HT1B agonists (8-OH-DPAT and CP-94253 respectively), in addition to L-dopa in the 6-OHDA-lesioned rat naïve to drug treatment, resulted in significantly lower levels of dyskinesia manifestation after two weeks of treatment compared to an L-dopa only treatment group (Munoz *et al.*, 2008). This effect is likely due to an ability of 5-HT1 agonists to reduce dysregulated release of dopamine from serotonergic neurons and hence pre-synaptic action (Carta *et al.*, 2007). Chronic treatment with L-Stepholidine, a naturally occurring D_1 receptor agonist and D_2 antagonist, significantly reduced axial, limb and ALO AIMs induced by L-dopa from day 4 of co-administration (Mo *et al.*, 2010). A dual mechanism of D2 antagonistic activity and D1 partial agonistic activity is proposed to restore the balance between direct and indirect striatal output pathways inhibiting behavioural sensitization induced by chronic L-dopa treatment. L-Stepholidine also shows 5-HT1A agonistic activity further contributing to an anti-dyskinetic effect. It is possible to reduce L-dopa priming for AIMs in the 6-OHDA-lesioned rat model and hence it would appear that the nNOS inhibitors ARR17477 and 7-NI are insufficient to attenuate L-dopa induced changes underlying dyskinesia. As such, it seems nNOS is not involved in L-dopa-induced priming for AIMs.

4.4.4 The effect of chronic nNOS inhibition on ropinirole-induced AIMS

Akin to results for the AIMs expression studies described in Chapter 3, ropinirole induced lower levels of AIMs during chronic treatment compared to L-dopa, and AIMS also developed more gradually over the 22 day chronic treatment period often not emerging until later treatment days than for L-dopa. These findings are iin line with previous priming studies comparing ropinirole with L-dopa. Carta *et al.* (2008b)

showed no ALO AIMs to be induced by ropinirole on day 1 and only very mild AIMs by day 19 of repeated treatment, whilst L-dopa induced mild ALO AIMs already on day 1 severely worsening by day 19. These are similar patterns to those of the experiments presented here, although direct comparison is made difficult by a differing representation of AIMs scores and the dose of ropinirole being considerably higher at 5.0 mg/kg compared to the 0.2 mg/kg employed in the present study. However, this does suggest that increasing the ropinirole dose 25-fold has little bearing on priming for ropinirole-induced AIMs. Furthermore, Papathanou *et al.* (2011) showed a significantly shorter duration of ALO AIMs on the final day of chronic treatment with ropinirole compared to L-dopa and there was also a trend for a reduction in ALO total scores.

In a study by Ravenscroft *et al.* (2004), measurement of contralateral rotations displayed by 6-OHDA-lesioned rats following chronic ropinirole and L-dopa treatment were similar in intensity and duration, used at a comparable dose to that employed here. However comparison of these studies does depend on interpretation of rotational behaviour in the 6-OHDA-lesioned rat and whether it is truly representational of dyskinesia, as there is inconsistency between rotations and AIMs measured in response to dopaminergic treatment (Marin *et al.*, 2006). A 6-OHDA-lesioned mouse model of AIMs showed chronic ropinirole administration induced no ALO AIMs whereas L-dopa induced a gradual increase in ALO AIMs over two weeks of treatment (Lundblad *et al.*, 2005). This supports a greater dyskinesiogenic effect of L-dopa than ropinirole. There is a chance that ropinirole-induced AIMs may have further increased if chronic dosing was continued beyond 22 days in the present studies, although this duration is relatively long considering similar experiments using varied doses lasted between 15 and 21 days (Lundblad *et al.*, 2005; Carta *et al.*, 2008c; Papathanou *et al.*, 2011). Additionally scores on the final day of priming (day 22) are comparable to those seen in acute studies where animals were treated with ropinirole alone (See Chapter 3; section 3.3.4.2) despite the fact all animals were priorly primed with L-dopa in Chapter 3, suggesting continued priming with ropinirole would have had no significant effect.

There was no significant effect of ARR17477 treatment on ropinirole-induced axial, limb, orolingual, ALO or locomotive AIMs throughout the 22 day priming period. Meanwhile 7-NI significantly increased axial AIMs compared to ropinirole alone as measured by total and peak scores as well as duration of AIMs on day 4, and totals were also significantly increased on day 16. This tendency for 7-NI to increase AIMs was not observed in the final acute challenges, following drug washout, where all animals were treated with ropinirole alone. Hence combined with the fact that ARR17477 did not alter ropinirole-induced AIMs, this finding suggests that it is a property specific to 7-NI such as MAOB and/or eNOS effects leading to a relative increase in AIMs, as previously discussed (see sections 4.4.3 and 3.4.2). There was a trend for prior treatment with ARR17477 to increase the duration of orolingual AIMs in the final ropinirole challenges, which is difficult to explain but given the very low levels exhibited with vehicle the difference itself is minimal suggesting a non-important effect. No studies reported in the literature appear to have investigated effects of adjunctive agents on ropinirole-induced AIMs development.

However, as for L-dopa-induced priming for dyskinesia, nNOS is not critically involved in ropinirole-induced development of dyskinesia.

4.4.5 Conclusion

The findings presented in this chapter suggest that nNOS does not play a key role in the induction of dyskinesia as assessed by AIMs in the 6-OHDA-lesioned rat. *De novo* chronic treatment with L-dopa or ropinirole in combination with either ARR17477 or 7-NI affords no significant improvement in dyskinesia during chronic treatment or indeed following acute dopaminergic treatment alone after drug-washout.

Whilst nNOS inhibitors may not be advantageous in prevention or management of dyskinesia, another possibility is that the model itself is unsuitable. It is questionable how truly representative abnormal involuntary movements in the 6-OHDA-lesioned rat are of the complex choreic and dystonic elements of dyskinesia seen in the clinic (Bezard *et al.*, 2001). The onset of AIMs also typically occurs immediately after initiation of dopaminergic treatment, unlike the clinical scenario, whilst interpretation of AIMs may be hindered by rotational activity. Additionally the unilateral 6-OHDA-model employed in these experiments does not show overt symptoms of PD in the absence of dopaminergic treatment. Meanwhile, the long-established MPTP-treated primate model of PD strongly mimics the parkinsonian state and is considered to display abnormal movements closely resembling those apparent in the human disorder upon dopaminergic treatment, and these can be assessed using clinical rating scales with only minor adaptation (Schneider, 1989; Imbert *et al.*, 2000; Langston *et al.*, 2000). With these differences between behavioural models in mind, the next chapter will examine the effects of nNOS inhibition on dyskinesia in the MPTP-treated primate.

Chapter 5 : The effects of nNOS inhibitor treatment on expression and priming for dyskinesia in MPTP-treated primates

5.1 Introduction

In the previous chapter it was reported that nNOS inhibition was ineffective in reducing the induction of AIMs by *de novo* L-dopa or ropinirole treatment in 6-OHDA-lesioned rats, suggesting that nNOS is not involved in the development of dyskinesia in this model of PD. Additionally results from Chapter 3 indicate that nNOS is not a key factor in the expression of established dyskinesia in this rodent model. Whilst these studies suggest nNOS and NO do not play an important role in these motor abnormalities, given the strong association between NO and basal ganglia function whereby glutamatergic activity can lead to NO synthesis and influence synaptic plasticity, the malfunctioning of which has already been linked to dyskinesia (Garthwaite *et al.*, 1989; Calabresi *et al.*, 2000; Chase & Oh, 2000; West & Grace, 2000)(see Chapter 1, section 1.4.4), perhaps the 6-OHDA-lesioned rat is not the most appropriate model to be using.

The assessment of AIMs as a measure of dyskinesia is a relatively recent advancement in animal modelling (Cenci *et al.*, 1998), and the behavioural repertoire of 6-OHDA-lesioned rats treated with dopaminergic drugs falls short of the motor abnormalities demonstrated by the longer-standing MPTP-treated primate (Jenner *et al.*, 1984; Langston *et al.*, 1984; Crossman, 1987). MPTP-treated primates commonly demonstrate chorea, dystonia, athetosis and akathisia following chronic dopaminergic treatment as well as rigidity, akinesia, bradykinesia and action tremor when off drug (Boyce *et al.*, 1990; Pearce *et al.*, 1995). Even the abnormal motor phenomena of an MPTP-treated primate off drug are easily noticeable whilst unilaterally 6-OHDA-lesioned rats show no overt symptoms of PD. The extent of motor disability can therefore be gauged simultaneously to dyskinesia in the primate model whereas this is not possible in the rat. Indeed rating scales for motor disability in primates tend to be based on clinical measures and have been developed in association with experienced neurologists in some laboratories including our own (Pearce *et al.*, 1998; Imbert *et al.*, 2000).

Upon dopaminergic medication 6-OHDA-lesioned rats can exhibit rotations and AIMs but these are arguably far removed from the characteristics of clinical PD. Obviously this disparity is partly due to the unilateral nature of toxin administration in the rat, but a full bilateral lesion is rarely tolerated (Simola *et al.*, 2007). Even the significance of rotations for anti-parkinsonian activity versus dyskinesiogenic potential of drugs is disputed (Lundblad *et al.*, 2002; Marin *et al.*, 2006). By contrast in the MPTP-treated primate locomotor activity is easily measured during treatment, which can provide additional insight into drug effects especially as it tends to positively correlate with dyskinesia (Kuoppamaki *et al.*, 2007).

The MPTP-treated primate model has been extensively investigated to date, with marmosets and other primates having basal ganglia anatomy more analogous to man than do rats, and displaying motor abnormalities frequently indistinguishable from those characterising idiopathic PD (Hardman *et al.*, 2002; Iravani & Jenner, 2011). Furthermore the MPTP model shows high predictive validity for translation of drugs treating PD into the clinic (Fox *et al.*, 2006), and all of the dopaminergic drugs used

for treatment of PD have proven effective in this model (Duty & Jenner, 2011). Thus this primate model certainly seems to mimic PD in many respects more closely than do rodents.

No studies to date have investigated the effect of nNOS inhibitors on dyskinesia in the MPTP-treated primate. The distinct advantages of the model over the 6-OHDA-lesioned rat, including its symptomatic and neurophysiological similarities to man, broader behavioural repertoire, proven clinical translation, and long-established validity, combined with the evidence for changes in NO in PD, afforded further investigation. In contrast with the studies undertaken thus far (Chapters 3 & 4), it is possible that nNOS inhibitors may demonstrate beneficial effects on expression and priming for dyskinesia in the MPTP-treated primate model.

5.1.1 Hypothesis

It is hypothesised that in MPTP-treated primates inhibition of nNOS will reduce the expression of established dyskinesia following dopaminergic drug treatment and also prevent the induction of dyskinesia from first L-dopa exposure.

5.1.2 Aims

The aim of the studies in this chapter was to determine whether, in MPTP-treated primates, nNOS inhibitors can attenuate the expression of dyskinesia following L-dopa or dopamine agonist treatment and also prevent development of dyskinesia following chronic L-dopa treatment. In order to achieve these aims the following shall be specifically investigated;

1. The effect of acute administration of ARR17477 in combination with L-dopa on dyskinesia expression in L-dopa-primed MPTP-treated primates

2. The effect of acute administration of ARR17477 in combination with ropinirole on dyskinesia expression in L-dopa-primed MPTP-treated primates

3. The effect of chronic nNOS inhibition on the induction of dyskinesia by L-dopa administration in MPTP-treated primates

5.2 Materials and methods

5.2.1 Introduction

The doses of ARR17477 required for nNOS inhibition in the acute and subsequent chronic *in vivo* studies in primates were based on *ex vivo* rodent studies described in Chapters 3 and 4. Following recovery from MPTP treatment, marmosets were primed to express dyskinesia by chronic dosing with L-dopa. These animals were then treated acutely with the pre-selected doses of ARR17477 in combination with L-dopa or ropinirole and assessed for locomotive activity, motor disability and dyskinesia. Next using a group of naïve MPTP-treated marmosets the effect of nNOS inhibition on L-dopa-induced priming for dyskinesia was investigated. The methods for these studies are described below.

5.2.2 Determination of the dose of nNOS inhibitor

5.2.2.1 ARR17477

For the dyskinesia expression studies the doses of ARR17477 (3, 6 and 12 mg/kg s.c.) selected were those employed in the rat studies of Chapter 3 (see section 3.2.3), which were all shown to significantly reduce nNOS activity *ex vivo* in agreement with published findings (O'Neill *et al.*, 2000; Reif *et al.*, 2000).

5.2.2.2 7-NI

Owing to home office requirements 7-NI could not be administered to marmosets using an i.p. route as previously used in the rat, hence a s.c. route was chosen and an *ex vivo* study was necessary to confirm inhibition of nNOS in the brain.

5.2.2.2.1 Animals

Male Wistar rats (200-250 g; Harlan, UK or B & K, UK) were housed 2-3 per cage in the Biological Service Unit, King's College London. Room temperatures were maintained at 19-21 ˚C at 55 % humidity with a 12 h light-dark cycle and animals had free access to pelleted food and water, as described in section 2.2.2. Experiments were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986 under Home Office project licence no. 70/6019 or 70/6898.

5.2.2.2.2 Ex vivo determination of 7-NI dose

Naïve male Wistar rats (n=4/group; 200-250 g) were treated with 7NI (20 mg/kg, suspended in peanut oil, s.c.) or vehicle (peanut oil, 1 ml/kg, s.c.). This dose was selected on the basis of the doses tested *ex vivo* in Chapter 3 and also in the published literature (Hantraye *et al.*, 1996; Przedborski *et al.*, 1996). Animals were culled by decapitation at 1 h after treatment and striata and cerebellum were dissected out, snap frozen and stored at -70 ˚C for measurement of nNOS activity by radioenzymatic assay.

5.2.2.2.3 Radioenzymatic measurement of NOS activity

nNOS activity was determined in brain homogenates by measuring enzymatic conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline as fully described in section 2.6.1. Briefly homogenised tissue samples were

centrifuged (as detailed in section 2.6.1.2), and supernatants transferred to a 96-well plate in triplicate. Boiled supernatant was used as a negative control. A pre-prepared reaction mixture containing L-[2,3,4-^{3}H] arginine monohydrochloride (1 mCi/ml; 16.7 µM) and $CaCl_2$ (6 mM) was added (30 µl/well) and the plates incubated for 1 h at 30 °C. The reaction was terminated and the reaction mixture further processed to measure L-[^{3}H]-citrulline formation, by the addition of resin and subsequent filtration of the samples as described in 2.6.1.2. L-[^{3}H]-citrulline was determined in a Beta-liquid scintiallation counter (see section 2.6.1.2) whilst protein content of samples was quantified with a NanoDrop spectrophotometer (see section 2.6.1.3). Data were analysed as described in section 2.6.1.4 expressing nNOS activity as L-[^{3}H]-citrulline formation per mg of protein per hour.

5.2.2.2.4 Effect of 7-NI on nNOS activity

7-NI (20 mg/kg s.c.) significantly reduced nNOS activity by 20 % in the striatum compared to vehicle treatment alone (**Figure 5-1b**). Meanwhile there was no significant change in nNOS activity in the cerebellum (**Figure 5-1a**).

5.2.3 Animals

Animals and husbandry are described fully in section 2.4.2. In brief, 16 adult common marmosets (*callithrax jacchus*; 10 female and 6 male; weight 320 to 450 g) were housed at a temperature of 25±1 °C with 50 % relative humidity on a 12 h light/dark cycle. All animals were fed fresh fruit once daily and had *ad libitum* access to Mazuri food pellets (Mazuri Primate Diet, Special Diet Services Ltd., UK) and water. Experiments were all conducted in accordance with Home Office regulations under the Animals (Scientific Procedures) Act 1986 and project license number 70/6345.

5.2.4 MPTP-treatment

As described in section 2.4.3-2.4.5, after a minimum acclimatisation period of 8 weeks marmosets were administered MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride; 2.0 mg/kg, in saline 0.9 %, s.c.) once daily for 5 consecutive days. Animals were then hand-fed for up to 4 months until able to feed independently and maintain a stable body weight. Following recovery from the acute effects of the toxin, MPTP-treatment induced a stable parkinsonian syndrome characterised by bradykinesia, akinesia, rigidity and hypophonia.

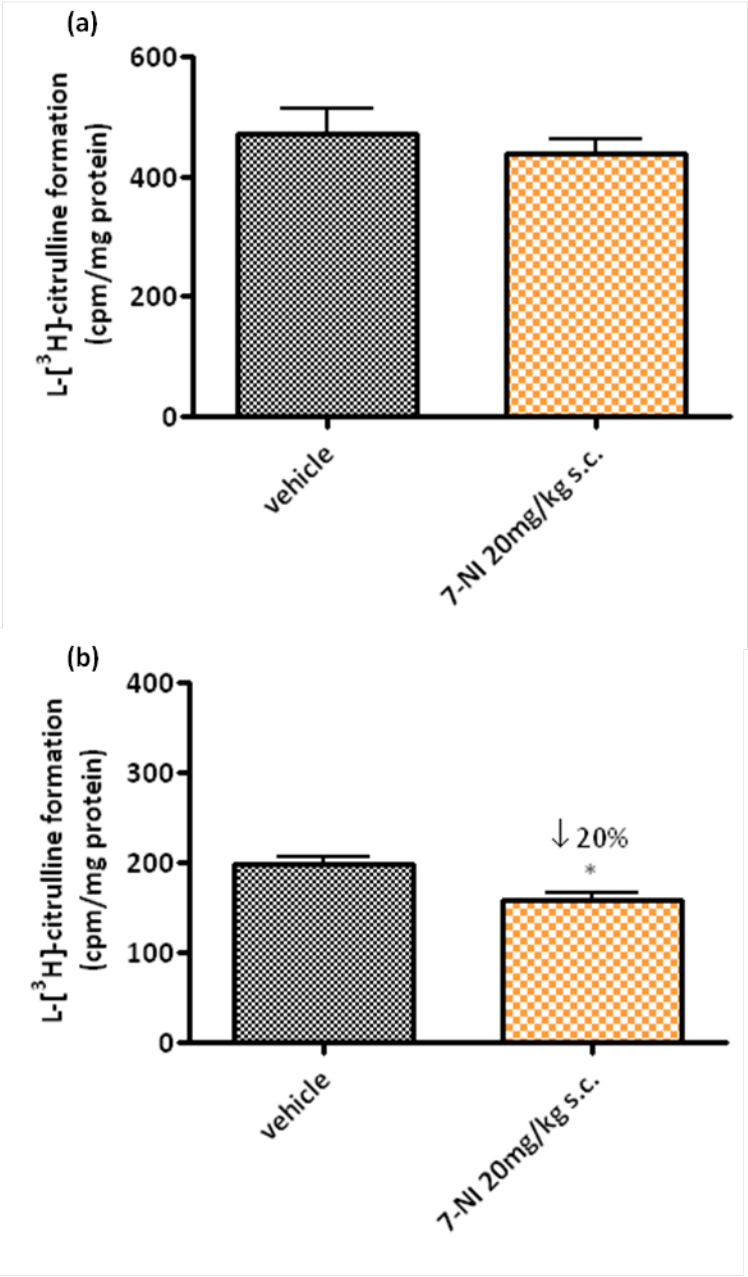

Figure 5-1 Radioenzymatic measurement of the effect of 7-NI (20 mg/kg s.c.) on nNOS activity in cerebellum and striatum. Naïve rats were treated with 7-NI or vehicle (peanut oil s.c.) and culled by decapitation at 1 h. Tissue samples were obtained from **(a)** cerebellum and **(b)** striatum. nNOS activity is displayed as L-[3H]-citrulline formation (cpm/mg protein); Data are presented as means ± SEM (n=4/group); *P<0.05 compared to vehicle treatment. Data were analysed by t-test.

5.2.5 Expression studies

5.2.5.1 Establishing L-dopa-induced dyskinesia

After recovery from the MPTP treatment, animals (n=6) were treated daily with L-dopa methyl ester (12.5 mg/kg + carbidopa 12.5 mg/kg p.o.) for a period of 8 weeks, to establish stable dyskinesia as described in section 2.5.1. Dyskinesia was assessed once-twice weekly in addition to other behavioural parameters (see section 5.2.7). Only animals displaying marked dyskinesia at the end of the priming period were taken forward to the expression studies.

5.2.5.2 L-dopa - dyskinesia expression studies

L-dopa-primed MPTP-treated marmosets (n=6) were pre-treated with carbidopa (12.5 mg/kg, in sucrose 10 % as a suspension, p.o.) followed 1 h later by L-dopa methyl ester (12.5 mg/kg free base, in sucrose 10 %, p.o.), plus concomitant ARR17477 (3, 6 or 12 mg/kg in 0.9 % saline s.c) or vehicle (0.9 % saline s.c). The L-dopa dose was chosen from preliminary studies showing 12.5 mg/kg p.o. gives a maximal response in terms of locomotor activity counts, motor disability reversal and dyskinesia scores (see section 2.5.5). ARR17477 or vehicle was administered according to a modified latin-square such that each animal received all treatments (repeated measures) in a randomised design (see Appendix, **Figure 0-2**). Animals were assessed behaviourally on each treatment day, and a minimum 1 week washout period was allocated between each treatment.

5.2.5.3 Ropinirole - dyskinesia expression studies

L-dopa-primed MPTP-treated marmosets (n=6) were pre-treated with domperidone (2 mg/kg in sucrose 10 %, p.o.) followed 1 h later by ropinirole (0.2 mg/kg, in sucrose 10 %, p.o.), plus concomitant ARR17477 (3, 6 or 12 mg/kg in 0.9 % saline s.c) or vehicle (0.9 % saline s.c). The ropinirole dose was chosen from preliminary studies showing 0.2 mg/kg p.o. is equipotent to the L-dopa dose employed with respect to locomotor activity counts, motor disability reversal and dyskinesia scores (see section 2.5.5). ARR17477 or vehicle was administered according to a modified latin-square such that each animal received all treatments (repeated measures) in a randomised manner (see Appendix, **Figure 0-2**). Animals were assessed behaviourally on each treatment day, and a minimum 1 week washout period was allocated between each treatment.

5.2.6 Priming study

Following recovery from MPTP-treatment, animals (n=10) were assessed for basal locomotor activity (see section 2.5.2) and assigned to one of two treatment groups, each matched for mean basal locomotor activity. All animals were administered L-dopa methyl ester (12.5 mg/kg + carbidopa 12.5 mg/kg, in sucrose 10 %, p.o.) once daily for 8 weeks except on behaviour assessment days where carbidopa was administered 1 h prior to L-dopa. Animals in Group 2 were also treated with 7-NI (20 mg/kg; in peanut oil, s.c.) dosed 30 min before L-dopa during weeks 1-4 and ARR17477 (1mg/kg; in 0.9 % saline, s.c.), dosed concomitantly with L-dopa during weeks 5-8. In Group 1 animals were also treated

with vehicle (1 ml/kg, s.c.; peanut oil, weeks 1-4 or 0.9 % saline, weeks 5-8). It became necessary to change the nNOS inhibitor from 7-NI to ARR17477 after the initial 4 weeks of chronic treatment as animals began to develop sores at the 7-NI/vehicle (s.c.) injection sites. ARR17477 has the advantage of being soluble in 0.9 % saline and produced no irritation. Animals were assessed behaviourally once or twice per week throughout the priming period (see **Figure 5-2**).

One week following withdrawal of chronic drug treatment, animals were challenged on two separate occasions one week apart with L-dopa methyl ester (10 mg/kg + benserazide 10 mg/kg, in 0.9 % saline, s.c.), and assessed for behaviour.

5.2.7 Behavioural assessment

Animals were placed in the behavioural test units following administration of carbidopa where necessary. Behavioural assessment was performed for 1 h before and up to 6 h after L-dopa/ropinirole/vehicle administration, as described in section 2.5. In brief, locomotor activity, motor disability and dyskinesia were monitored. Locomotor activity was measured by infrared beam interruptions which accumulated and recorded electronically in 10 and 30 min time bins. Animals were also observed and scored for motor disability and dyskinesia during the last 10 min of consecutive 30 min intervals. Full descriptions of assessment criteria are provided in sections 2.5.2 - 2.5.5.

5.2.8 Data and statistical analysis

For both expression and priming studies total activity/scores over the assessment period were calculated by AUC (Graphpad Prism version 5.0) using the trapezoid method where each successive 30 min was labelled as a single time-bin. Peak activity/score was taken as the maximum count/score achieved per 30 min within the full assessment period. 'On-time' for locomotor activity was defined as the duration of the test period that an animal exhibited activity greater than 2x its mean baseline count calculated from 10 min time-bin data. 'On-time' for reversal of motor disability was defined as the duration of the test period that an animal scored below 8 on the motor disability scale and 'on-time' for dyskinesia was defined as the duration of the test period that an animal scored above 0 on the dyskinesia rating scale. In all cases statistical significance was set at $p<0.05$ and analyses were carried out in Graphpad Prism 5. Additionally a 'trend' was described in the data where there was greater than a 75 % change from vehicle treated animals.

For expression studies time-course data were plotted as medians at 30 min intervals over 6 h following drug administration, with pre-treatment baseline plotted at t=0. Drugs were administered at t=0 on graphs. Time-course data for locomotor activity, motor disability and dyskinesia were analysed by 2-way ANOVA followed by Friedman's and Dunn's post hoc test where appropriate. Total and peak activity/scores, and on-time, were analysed by Friedman's and Dunn's post hoc test.

For the priming studies median total and peak activity/scores, and on-time, were plotted from weekly test days using data collected over a 4 h period following treatment. Pre-treatment baseline data were plotted at week 0 where animals were drug naïve. Data were analysed by 2-way ANOVA followed by Kruskall-Wallis or Friedman's test and then Dunn's post hoc test where appropriate. Summed data were also plotted for the full priming period using AUC (Graphpad Prism version 5.0) of 8 week total and peak activity/scores, and on-time. Mann-Whitney tests were used to compare the effect of treatment on summed total activity/scores for the priming period and also the subsequent L-dopa challenges.

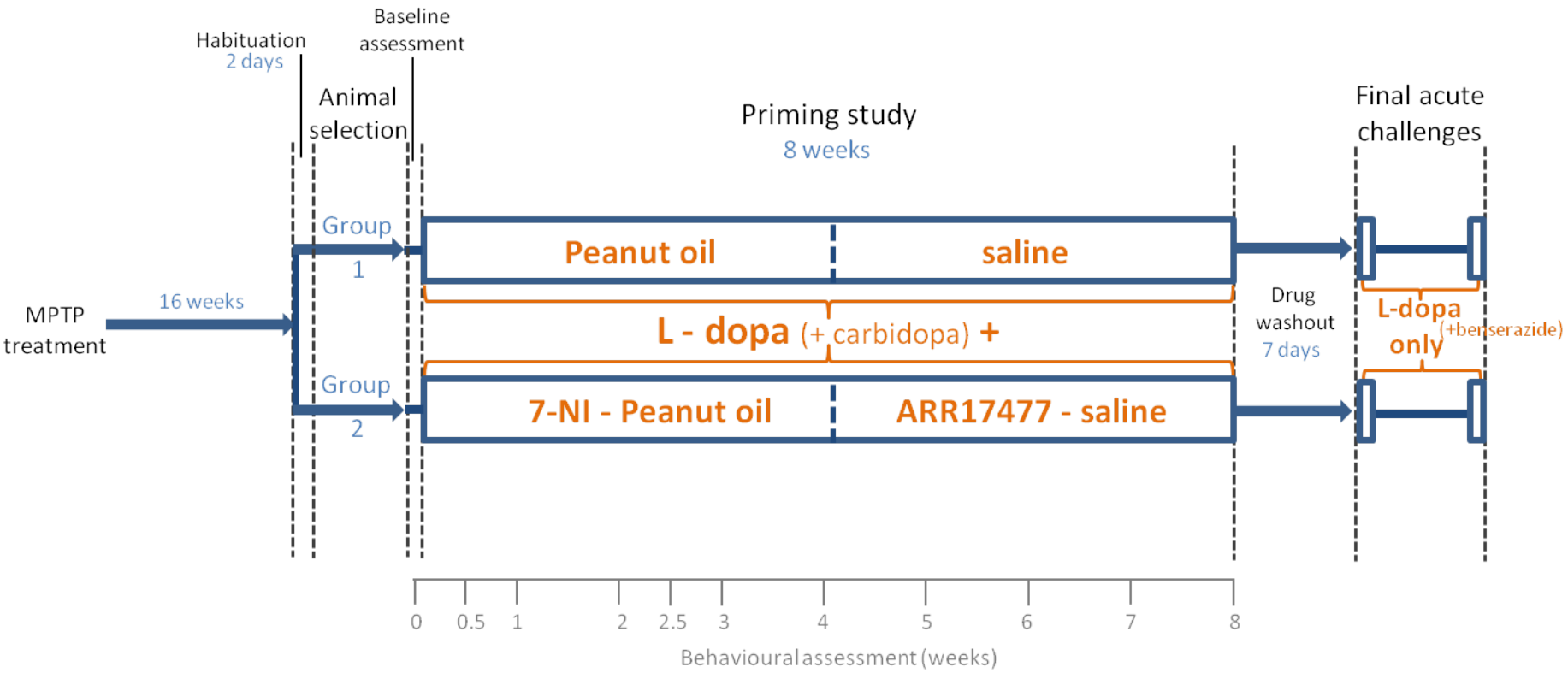

Figure 5-2 Summary time-line of animal groups and treatments for priming study. Group 1 were treated chronically with L-dopa (12.5 mg/kg + carbidopa 12.5 mg/kg p.o.) in combination with vehicle (1ml/kg, s.c.; peanut oil; weeks 1-4 or saline; weeks 5-8) and Group 2 were treated chronically with L-dopa (12.5 mg/kg + carbidopa 12.5 mg/kg p.o.) in combination with 7-NI (20 mg/kg, s.c.; in peanut oil; weeks 1-4) or ARR17477 (1mg/kg, s.c.: in saline; weeks 5-8). Both groups were treated with L-dopa (10.0 mg/kg + benserazide 10.0 mg/kg s.c.) in final acute challenges.

5.3 Results

5.3.1 The effect of ARR17477 on L-dopa-induced locomotor activity, motor disability and dyskinesia expression in L-dopa-primed MPTP-treated marmosets

Vehicle-vehicle administration did not alter locomotor activity, motor disability or dykinesia expression over time (**Figure 5-3a, Figure 5-4a** & **Figure 5-5a**).

5.3.1.1 Locomotor activity

-nNOS inhibitor + vehicle

ARR17477 (12 mg/kg s.c.) plus vehicle did not alter locomotor activity in MPTP-treated marmosets compared to vehicle-vehicle treatment (**Figure 5-3a**). This lack of effect was confirmed by unchanged total locomotor activity counts (median counts of 60 for both ARR17477 plus vehicle and vehicle-vehicle groups), peak locomotor activities (120 counts/30 min) and on-times (20 min) for ARR17477 plus vehicle and vehicle-vehicle groups (**Figure 5-3b**, **c** & **d**).

-Vehicle + L-dopa

L-dopa alone gradually increased locomotor activity from 30 min reaching a maximum of 400 counts/30 min at 90 min, returning to baseline levels by 120 min (**Figure 5-3a**). Total locomotor activity over 6 h was 1100 counts which was significantly higher than vehicle-vehicle treated animals (Figure 5.2b). Peak locomotor activity of 500 counts and on-time of 140 min was achieved following L-dopa alone treatment (**Figure 5-3b** & **c**).

-nNOS inhibitor + L-dopa

Although there was no significant effect of ARR17477 (3, 6 or 12 mg/kg) on L-dopa-induced increase in locomotor activity as measured by total counts (**Figure 5-3b**), peak activity (**Figure 5-3c**) and on-time (**Figure 5-3d**), total activity tended to be greater following all doses of ARR17477, and peak activity tended to be greater after ARR17477 (12 mg/kg), compared to L-dopa alone.

5.3.1.2 Motor disability

-nNOS inhibitor + vehicle

ARR17477 (12 mg/kg) alone did not reverse motor disability with scores not significantly different from vehicle-vehicle treatment (**Figure 5-4a**). Similarly there was no significant effect of ARR17477 alone on total motor disability score (ARR17477-vehicle=6, vehicle-vehicle=15), peak motor disability score (ARR17477-vehicle=10, vehicle-vehicle=11), or on-time (both 0 min), (**Figure 5-4b-d**).

-Vehicle + L-dopa

L-dopa alone induced an expected reversal of motor disability compared to vehicle-vehicle treatment with scores of 2 from 30-120 min after administration followed by a gradual return to baseline levels from 150-240 min (**Figure 5-4a**). Overall total reversal of motor disability had a median score of 75 (**Figure 5-4b**), with a peak score of 2 and on-time of 180 min (**Figure 5-4c & d**). Both peak motor disability score and on-time were significantly greater than for vehicle-vehicle treatment. The total change in motor disability score over 6 h tended to be greater than vehicle-vehicle, although it was not statistically significant (**Figure 5-4b**).

-nNOS inhibitor + L-dopa

There was no significant effect of ARR17477 (3, 6 or 12 mg/kg) on L-dopa-induced reversal of motor disability as measured by total change in motor disability score (**Figure 5-4b**), peak score (**Figure 5-4c**) and on-time (**Figure 5-4d**).

5.3.1.3 Dyskinesia

-nNOS inhibitor + vehicle

ARR17477 (12 mg/kg) plus vehicle treatment did not induce dyskinesia throughout the 6 h period of behavioural assessment, with scores not significantly different to vehicle-vehicle treatment (**Figure 5-5a**). Total and peak dyskinesia both scored 0, and no on-time was recorded for ARR17477 plus vehicle similar to vehicle-vehicle treatment groups (**Figure 5-5b-d**).

-Vehicle + L-dopa

L-dopa alone immediately induced dyskinesia expression with a score of 2 at 30 min, increasing to 3 between 90 and 120 min and returning to a score of 0 between 150-180 min (**Figure 5-5a**). Total dyskinesia scored was 10, with a peak score of 3 and on-time of 180 min (**Figure 5-5b-d**). Total and peak dyskinesia, and on-time all tended to be greater than for vehicle-vehicle treatment, although these effects were not statistically significant.

-nNOS inhibitor + L-dopa

There was no significant effect of ARR17477 (3, 6 or 12 mg/kg) on L-dopa-induced dyskinesia as measured by total dyskinesia score (**Figure 5-5b**), peak score (**Figure 5-5c**) and on-time (**Figure 5-5d**).

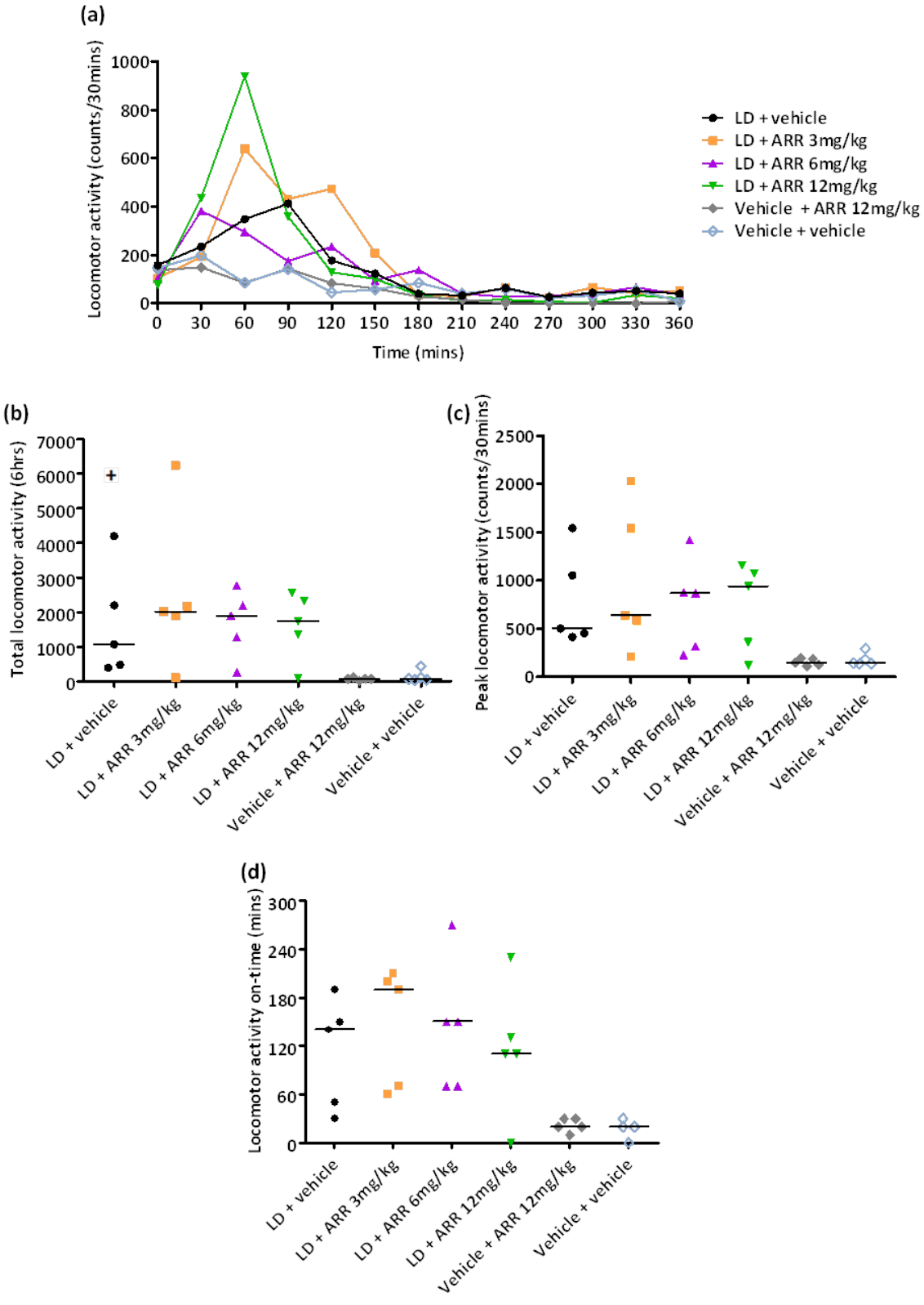

Figure 5-3 Locomotor activity following ARR17477 plus L-dopa treatment. ARR17477 (ARR; 3, 6 or 12 mg/kg s.c) and L-dopa (LD; 12.5 mg/kg plus carbidopa 12.5 mg/kg p.o.) treatment in L-dopa-primed MPTP-treated marmosets. Data are presented as medians (n=5); **(a)** Time-course, and also individual values **(b)** Total, **(c)** Peak and **(d)** On-time. +$p<0.05$ compared to vehicle + vehicle treatment. Time-course data were analysed by 2-way-ANOVA and all other data were analysed by Friedman's test followed by Dunn's post hoc test.

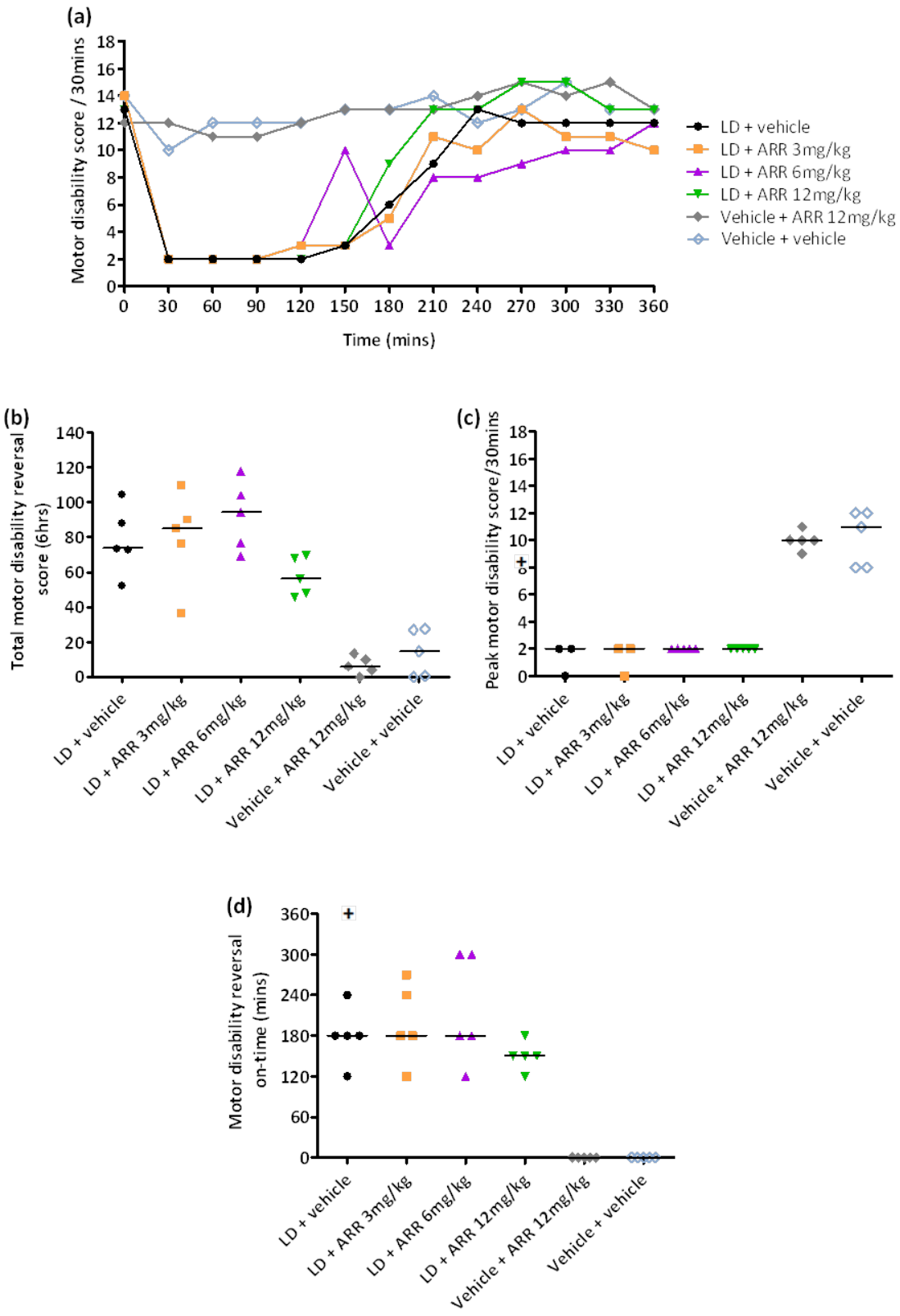

Figure 5-4 Motor disability following ARR17477 plus L-dopa treatment. ARR17477 (ARR; 3, 6 or 12 mg/kg s.c) and L-dopa (LD; 12.5 mg/kg plus carbidopa 12.5 mg/kg p.o.) treatment in L-dopa-primed MPTP-treated marmosets. Data are presented as medians (n=5); **(a)** Time-course, and also individual values **(b)** Total, **(c)** Peak and **(d)** On-time. +p<0.05 compared to vehicle + vehicle treatment. Time-course data were analysed by 2-way-ANOVA and all other data were analysed by Friedman's test followed by Dunn's post hoc test.

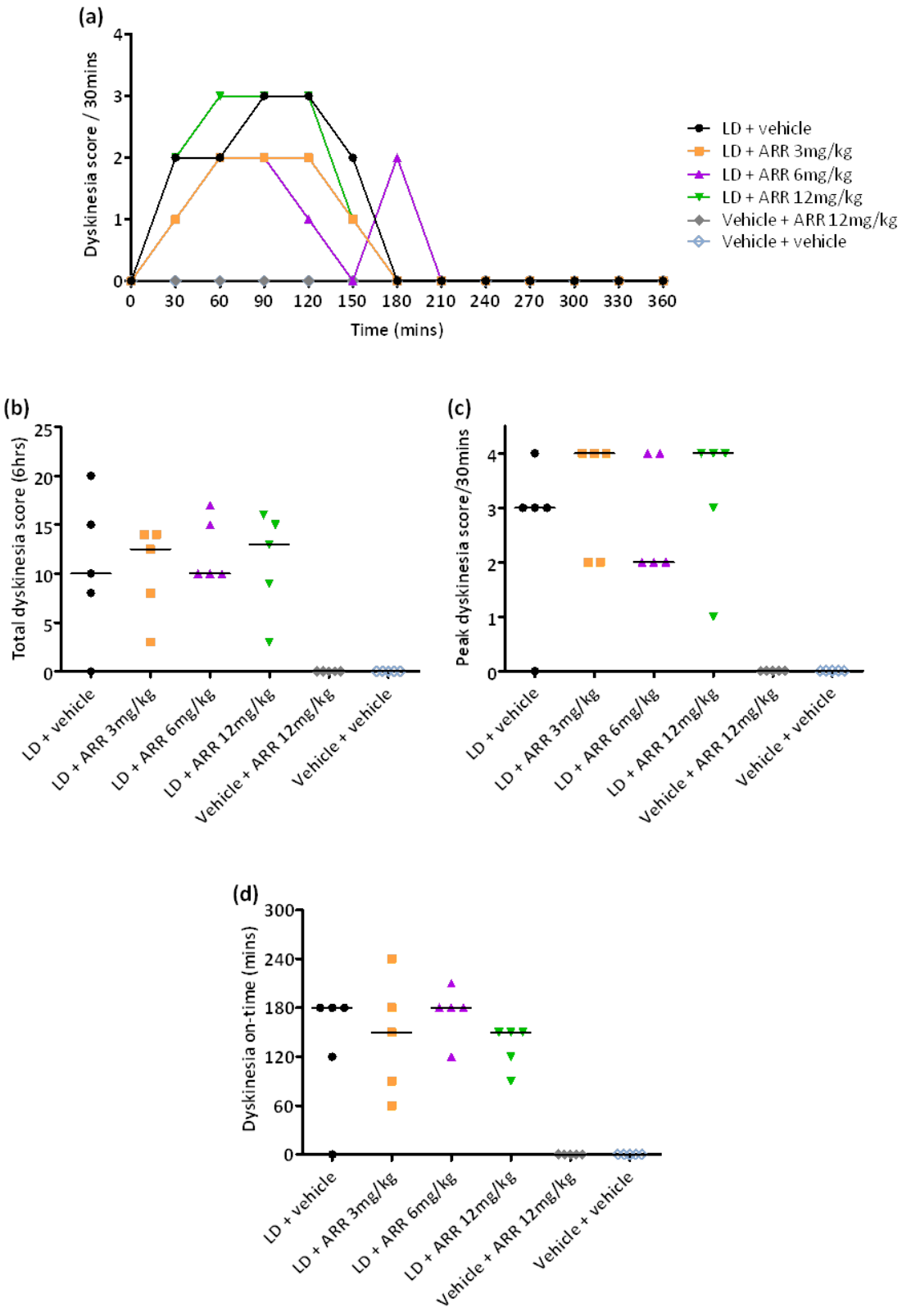

Figure 5-5 Dyskinesia expression following ARR17477 plus L-dopa treatment. ARR17477 (ARR; 3, 6 or 12 mg/kg s.c) and L-dopa (LD; 12.5 mg/kg plus carbidopa 12.5 mg/kg p.o.) treatment in L-dopa-primed MPTP-treated marmosets. Data are presented as medians (n=5); **(a)** Time-course, and also individual values **(b)** Total, **(c)** Peak and **(d)** On-time. +p<0.05 compared to vehicle + vehicle treatment. Time-course data were analysed by 2-way-ANOVA and all other data were analysed by Friedman's test followed by Dunn's post hoc test.

5.3.2 The effect of ARR17477 on ropinirole-induced locomotor activity, motor disability and dyskinesia expression in L-dopa-primed MPTP-treated marmosets

Vehicle-vehicle administration did not alter locomotor activity, motor disability or dykinesia expression over time (**Figure 5-6a, Figure 5-7a & Figure 5-8a**).

5.3.2.1 Locomotor acticity

-nNOS inhibitor + vehicle

ARR17477 (12 mg/kg) plus vehicle treatment did not alter locomotor activity in MPTP-treated marmosets compared to vehicle-vehicle treatment (**Figure 5-6a**). This was confirmed by matching total locomotor activity counts (median counts of 25 for both ARR17477 plus vehicle and vehicle-vehicle groups), peak locomotor activities (150 counts/30 min) and on-times (20 min) for ARR17477 alone and vehicle-vehicle groups (**Figure 5-6b, c & d**).

-Vehicle + ropinirole

Ropinirole alone increased locomotor activity from 30 min with a maximum effect of 700 counts/30 min at 90 min, returning to baseline levels by 150 min with small fleeting amounts of activity thereafter (**Figure 5-6a**). Total locomotor activity recorded over the 6 h was 2300 counts, with a peak activity of 700counts/30 min and on-time measuring 150 min (**Figure 5-6b**, **c** & **d**). Total, peak and on-time for ropinirole-induced locomotor activity tended to be greater than in vehicle-vehicle treated animals, although the difference was not statistically significant.

-nNOS inhibitor + ropinirole

There was no significant effect of ARR17477 (3, 6 or 12 mg/kg) on ropinirole-induced increase in locomotor activity as measured by total counts (Figure 5-6b), peak activity (Figure 5-6c) and on-time (Figure 5-6d), compared to ropinirole alone.

5.3.2.2 Motor disability

-nNOS inhibitor + vehicle

ARR17477 (12 mg/kg) alone did not reverse motor disability with scores not significantly different from vehicle-vehicle treatment (**Figure 5-7a**). ARR17477 alone produced no significant change in total motor disability score compared to vehicle-vehicle (both scored 8), the peak motor disability score (both 10), or on-time (both 0 min), (**Figure 5-7b-d**).

-Vehicle + ropinirole

Ropinirole alone significantly reversed motor disability compared to vehicle-vehicle treatment with scores of 5 at 30 min, further improving to a score of 2 at 60-120 min and returning gradually to baseline

levels between 240 and 300 min (**Figure 5-7a**). Overall total reversal of motor disability had a median score of 65 (**Figure 5-7b**), with a peak motor disability score of 2 and on-time of 150 min (**Figure 5-7c** & **d**). Both peak motor disability score and on-time were significantly greater than for vehicle-vehicle treatment.

-nNOS inhibitor + ropinirole

ARR17477 (3, 6 or 12 mg/kg) had no significant effect on ropinirole-induced reversal of motor disability as measured by total score (**Figure 5-7b**), peak score (**Figure 5-7c**) and on-time (**Figure 5-7d**).

5.3.2.3 Dyskinesia

-nNOS inhibitor + vehicle

ARR17477 (12 mg/kg) alone did not induce dyskinesia throughout the 6 h of behavioural assessment, showing no difference to vehicle-vehicle dyskinesia scores (**Figure 5-8a**). Total and peak dyskinesia both scored 0, and no on-time was recorded for the ARR17477 alone or vehicle-vehicle treatment groups (**Figure 5-8b-d**).

-Vehicle + ropinirole

Ropinirole alone induced dyskinesia expression with a score of 1.5 at 60 min, increasing to 2 at 90 min and declining thereafter reaching 0 by 180 min (**Figure 5-8a**). Total dyskinesia scored over the 6 h was 6.5, with a peak score of 2 and on-time of 90 min (**Figure 5-8b-d**). Total and peak dyskinesia, and on-time tended to be greater than for vehicle-vehicle treatment, although this difference was not statistically significant.

-nNOS inhibitor + ropinirole

There was no significant effect of ARR17477 (3, 6 or 12 mg/kg) on ropinirole-induced dyskinesia as measured by total score (**Figure 5-8b**), peak score (**Figure 5-8c**) and on-time (**Figure 5-8d**).

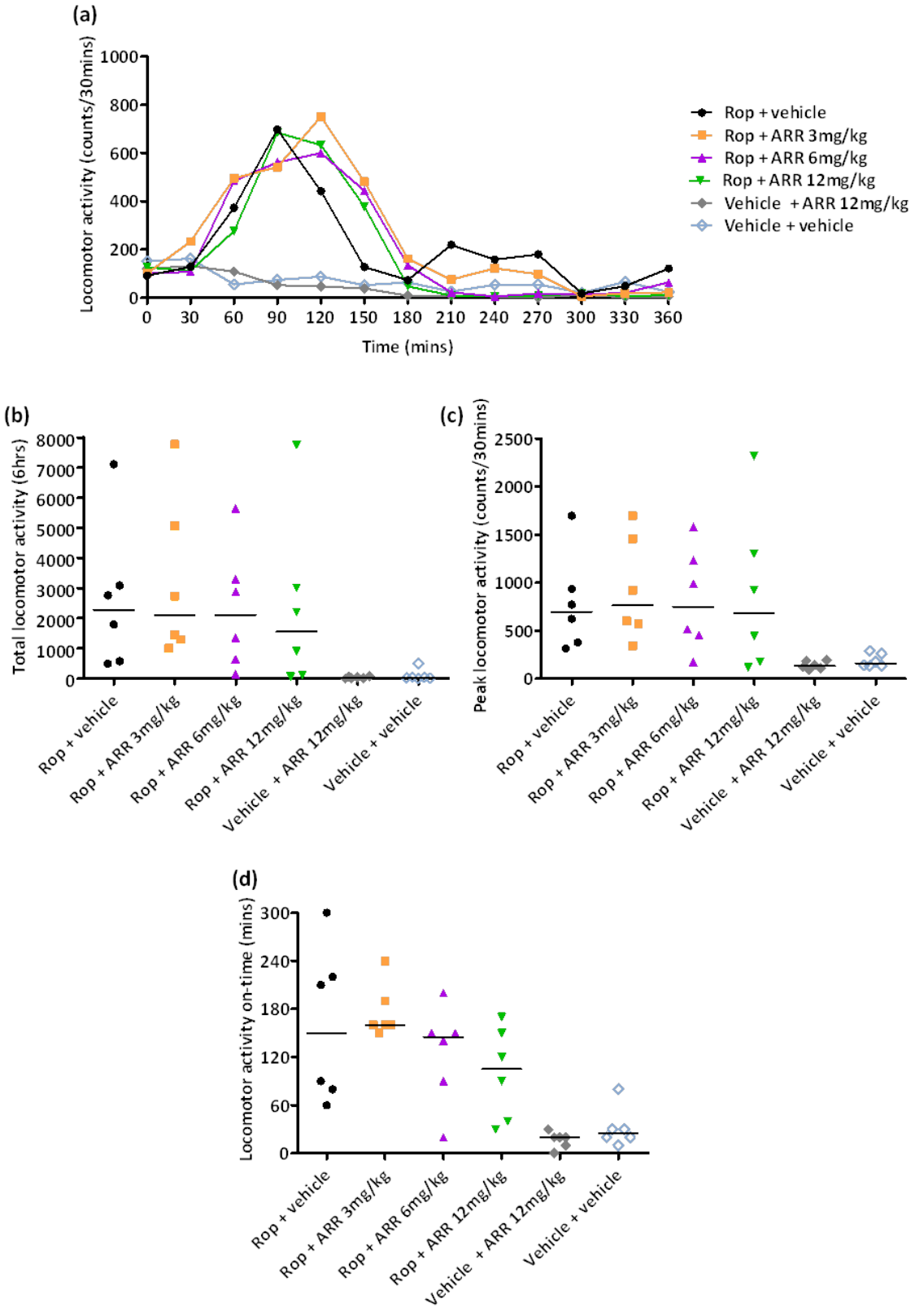

Figure 5-6 Locomotor activity following ARR17477 plus ropinirole treatment. ARR17477 (ARR; 3, 6 or 12 mg/kg s.c) and ropinirole (Rop; 0.2 mg/kg plus domperidone 2 mg/kg p.o.) treatment in L-dopa-primed MPTP-treated marmosets. Data are presented as medians (n=6); **(a)** Time-course, and also individual values **(b)** Total, **(c)** Peak and **(d)** On-time. Time-course data were analysed by 2-way-ANOVA and all other data were analysed by Friedman's test.

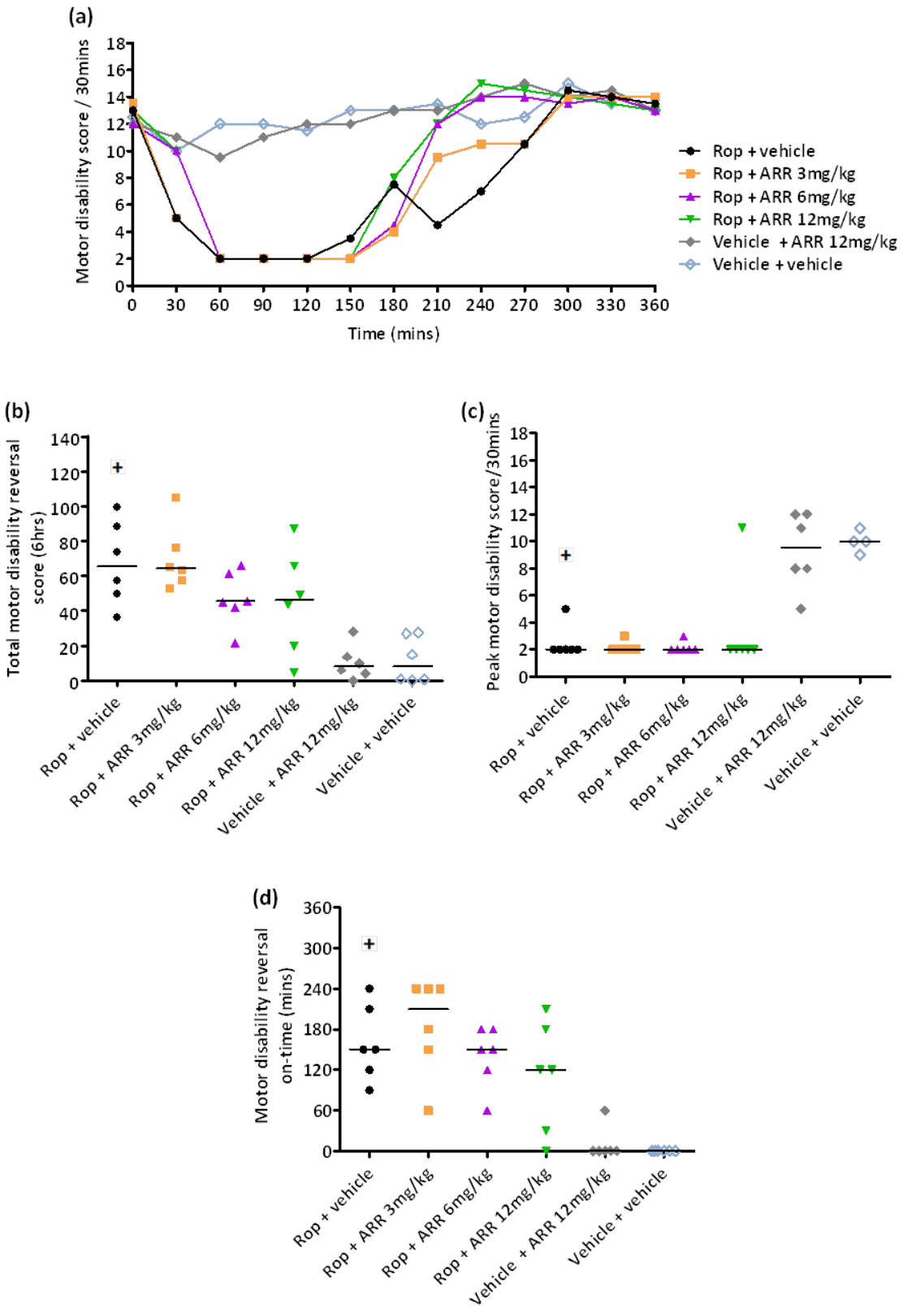

Figure 5-7 Motor disability following ARR17477 plus ropinirole treatment. ARR17477 (ARR; 3, 6 or 12 mg/kg s.c) and ropinirole (Rop; 0.2 mg/kg plus domperidone 2 mg/kg p.o.) treatment in L-dopa-primed MPTP-treated marmosets. Data are presented as medians (n=6); **(a)** Time-course, and also individual values **(b)** Total, **(c)** Peak and **(d)** On-time. +p<0.05 compared to vehicle + vehicle treatment. Time-course data were analysed by 2-way-ANOVA and all other data were analysed by Friedman's test followed by Dunn's post hoc test.

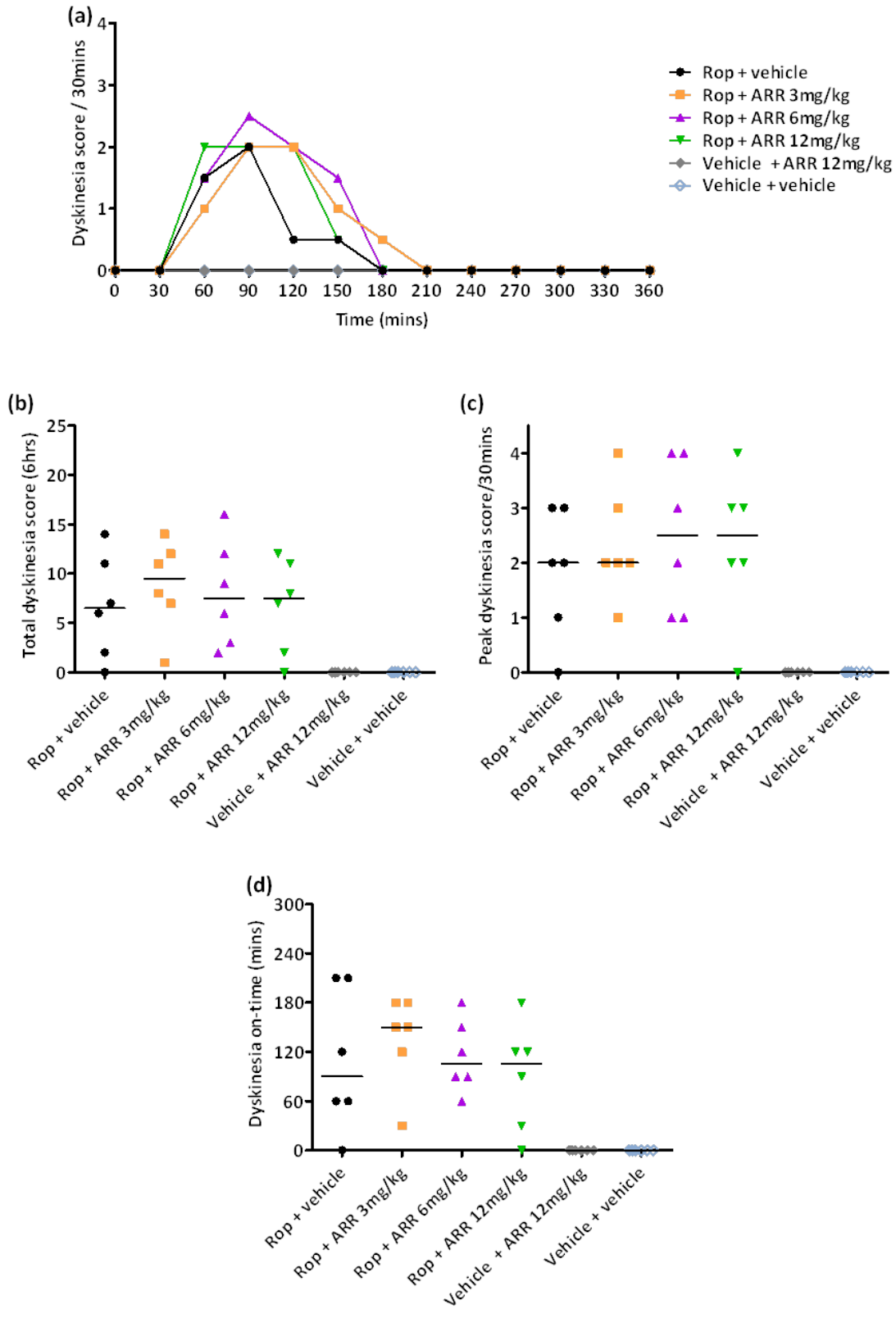

Figure 5-8 Dyskinesia expression following ARR17477 plus ropinirole treatment. ARR17477 (ARR; 3, 6 or 12 mg/kg s.c) and ropinirole (Rop; 0.2 mg/kg plus domperidone 2 mg/kg p.o.) treatment in L-dopa-primed MPTP-treated marmosets. Data are presented as medians (n=6); **(a)** Time-course, and also individual values **(b)** Total, **(c)** Peak and **(d)** On-time. Time-course data were analysed by 2-way-ANOVA and all other data were analysed by Friedman's test.

5.3.3 The effect of nNOS inhibition on locomotor activity, motor disability and dyskinesia induction following *de novo* chronic treatment with L-dopa plus nNOS inhibitor in MPTP-treated marmosets

5.3.3.1 Locomotor activity

-Vehicle + L-dopa

In week 1 of chronic treatment L-dopa-induced an expected increase in locomotor activity showing 800 counts/4 h, increasing to a total of 2300 counts/4 h by week 3 (**Figure 5-9a**). Although activity did not reach this level again, locomotor activity remained raised around 700 counts/4 h from week 4 onwards. In week 1 peak locomotor activity was 450 counts/30 min increasing to a maximal peak in week 3 of 650 counts/30 min after which levels remained stable with a peak activity of around 300 counts/30 min (**Figure 5-9b**). As expected all parameters significantly increased over the 8 week period (**Figure 5-9a-b**).

-nNOS inhibitor + L-dopa

There was no significant effect of nNOS inhibition on L-dopa-induced locomotor activity over the 8 week chronic administration phase as measured by total and peak activity (**Figure 5-9a-b**). These findings are further supported by the summed total and peak locomotor activity (**Figure 5-9a'-b'**) showing no significant difference between the two treatment groups.

5.3.3.2 Motor disability

-Vehicle + L-dopa

L-dopa reversed motor disability giving a total score of 30 over 4 h in week 1 (**Figure 5-10a**). Total scores then remained stable between 30 (week 4) and 50 (week 7). Maximum reversal of motor disability was achieved in week 1 (score 2.5) and remained controlled throughout the chronic treatment period with maximum scores of 2.5-4 (**Figure 5-10b**). The on-time for motor disability reversal gradually increased from 1 h in week 1 to a maximal on-time of 3 h in weeks 6-8 (**Figure 5-10c**). Reversal of motor disability significantly increased over the course of the chronic treatment period in terms of total score, peak score and on-time (**Figure 5-10a-c**).

-nNOS inhibitor + L-dopa

There was no significant effect of nNOS inhibition on L-dopa-induced motor disability reversal over the 8 week chronic administration phase as measured by total activity, peak activity and on-time (**Figure 5-10a-c**). These findings are further supported by summed total, peak and on-time for motor diability (**Figure 5-10a'-c'**) showing no significant difference compared to L-dopa alone.

5.3.3.3 Dyskinesia

-Vehicle + L-dopa

Dyskinesia gradually increased over time with a total score of 2.5 during 4 h in week 1 and reaching a maximum total score of 9 by week 7 (**Figure 5-11a**). The peak dyskinesia score also gradually increased from 1 in week 1 to a maximum peak score of 2 in weeks 5, 7 and 8 (**Figure 5-11b**). The duration of dyskinesia also increased over the treatment period with on-times of 75 min in week 1 reaching a maximum of 120-150 min in weeks 5, 7 and 8 (**Figure 5-11c**).

-nNOS inhibitor + L-dopa

There was no significant effect of nNOS inhibition on L-dopa-induced dyskinesia over the chronic treatment period as measured by total scores, peak scores and on-time (**Figure 5-11a-c**). These findings are substantiated by summed total, peak and on-time for dyskinesia (**Figure 5-11a'-c'**), showing no significant difference between the two treatment groups. However there was a tendency for nNOS inhibition to increase total and peak summed L-dopa-induced dyskinesia over the 8 weeks despite a lack of statistical significance (**Figure 5-11a'-b'**).

5.3.3.4 L-dopa final acute challenges

Following withdrawal of chronic treatment with L-dopa plus vehicle, subsequent acute challenge with L-dopa only induced an expected increase in locomotor activity with a median total of 1400 counts/4 h (**Figure 5-12a**), peak activity of 500 counts/30 min (**Figure 5-12a'**) and an on-time of 90 min (**Figure 5-12a''**). Similarly total motor disability scored 60 (**Figure 5-12b**), with a peak score of 2 (**Figure 5-12b'**) and on-time of 180 min (**Figure 5-12b''**), and total dyskinesia scored 13.5 (**Figure 5-12c**), with a peak score of 3 (**Figure 5-12c'**) and duration of 180 min (**Figure 5-12c''**).

Following withdrawal of chronic treatment with L-dopa plus concomitant nNOS inhibitor, challenge with L-dopa alone also produced an increase in locomotor activity, reversal of motor disability and expression of dyskinesia (**Figure 5-12a-c**). There was no significant difference in acute L-dopa response in animals who had received prior chronic treatment with L-dopa in the presence of the nNOS inhibitor compared to L-dopa alone for locomotor activity, motor disability or dyskinesia as measured by total or peak activity/scores, or on-time (**Figure 5-12a-a'', b-b" & c-c''**).

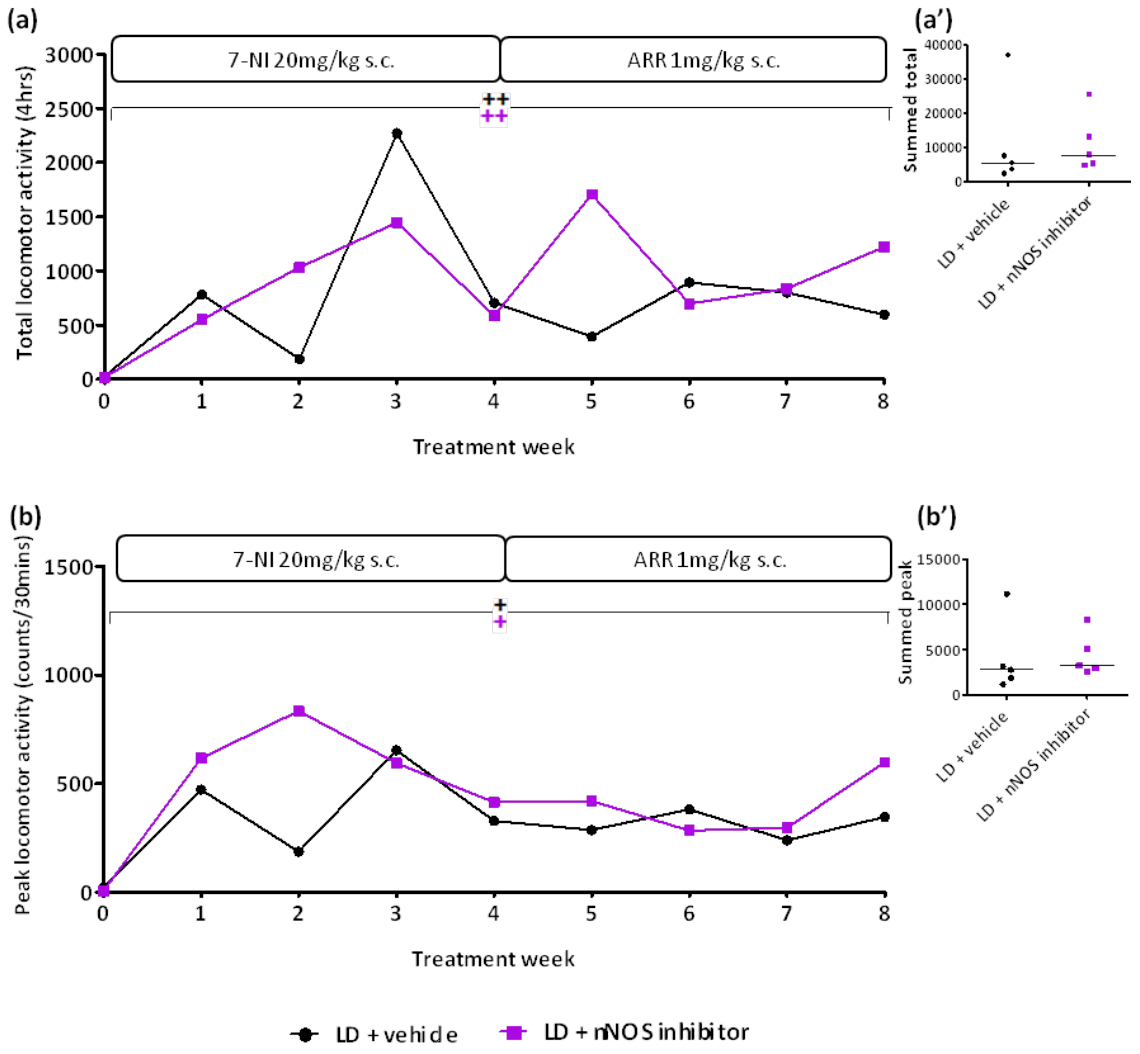

Figure 5-9 Locomotor activity following chronic treatment with nNOS inhibitor plus L-dopa. 7-NI (20 mg/kg s.c.; weeks 1-4) or ARR17477 (ARR; 1 mg/kg s.c; weeks 5-8) and L-dopa (LD; 12.5 mg/kg plus carbidopa 12.5 mg/kg p.o.) treatment in MPTP-treated marmosets, L-dopa naïve at week 0 (t=0 indicates baseline activity). Data are presented as medians (n=5); **(a)** Total and **(b)** Peak, and also individual values **(a')** Summed total and **(b')** Summed peak +p<0.05, ++p<0.01 for time (colours representative as in key). Weekly behavioural data were analysed by 2-way-ANOVA followed by Friedman's test for time. Summed data were analysed by Mann-Whitney tests.

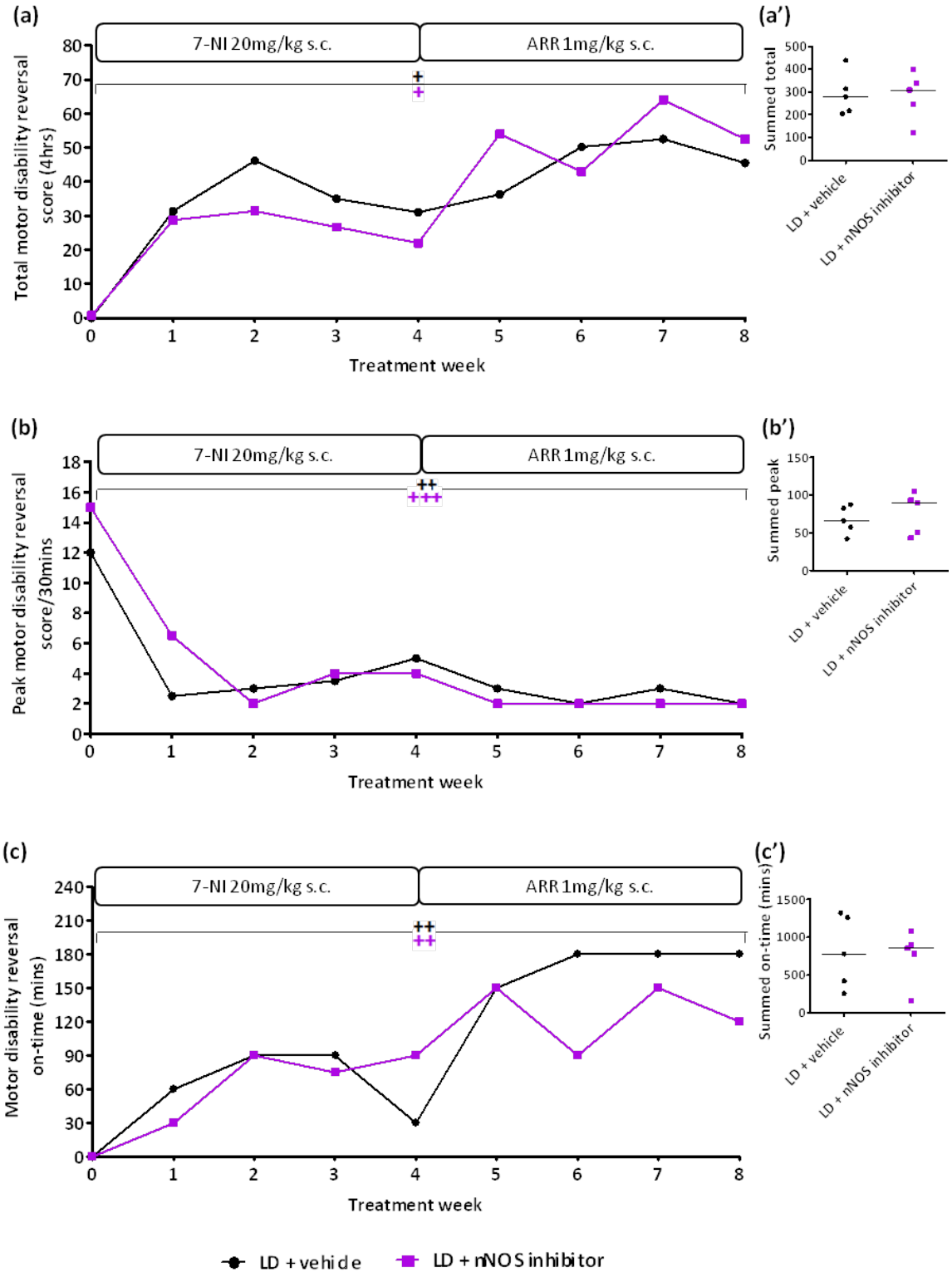

Figure 5-10 Motor Disability following chronic treatment with nNOS inhibitor plus L-dopa. 7-NI (20 mg/kg s.c.; weeks 1-4) or ARR17477 (ARR; 1 mg/kg s.c; weeks 5-8) and L-dopa (LD; 12.5 mg/kg mg/kg plus carbidopa 12.5 mg/kg p.o.) treatment in MPTP-treated marmosets, L-dopa naïve at week 0 (t=0 indicates baseline activity). Data are presented as medians (n=5); **(a)** Total, **(b)** Peak and **(c)** On-time, and also individual values **(a')** Summed total, **(b')** Summed peak and **(c')** Summed on-time +p<0.05, ++p<0.01, +++p<0.001 for time (colours representative as in key). Weekly behavioural data were analysed by 2-way-ANOVA followed by Friedman's test for time. Summed data were analysed by Mann-Whitney tests. .

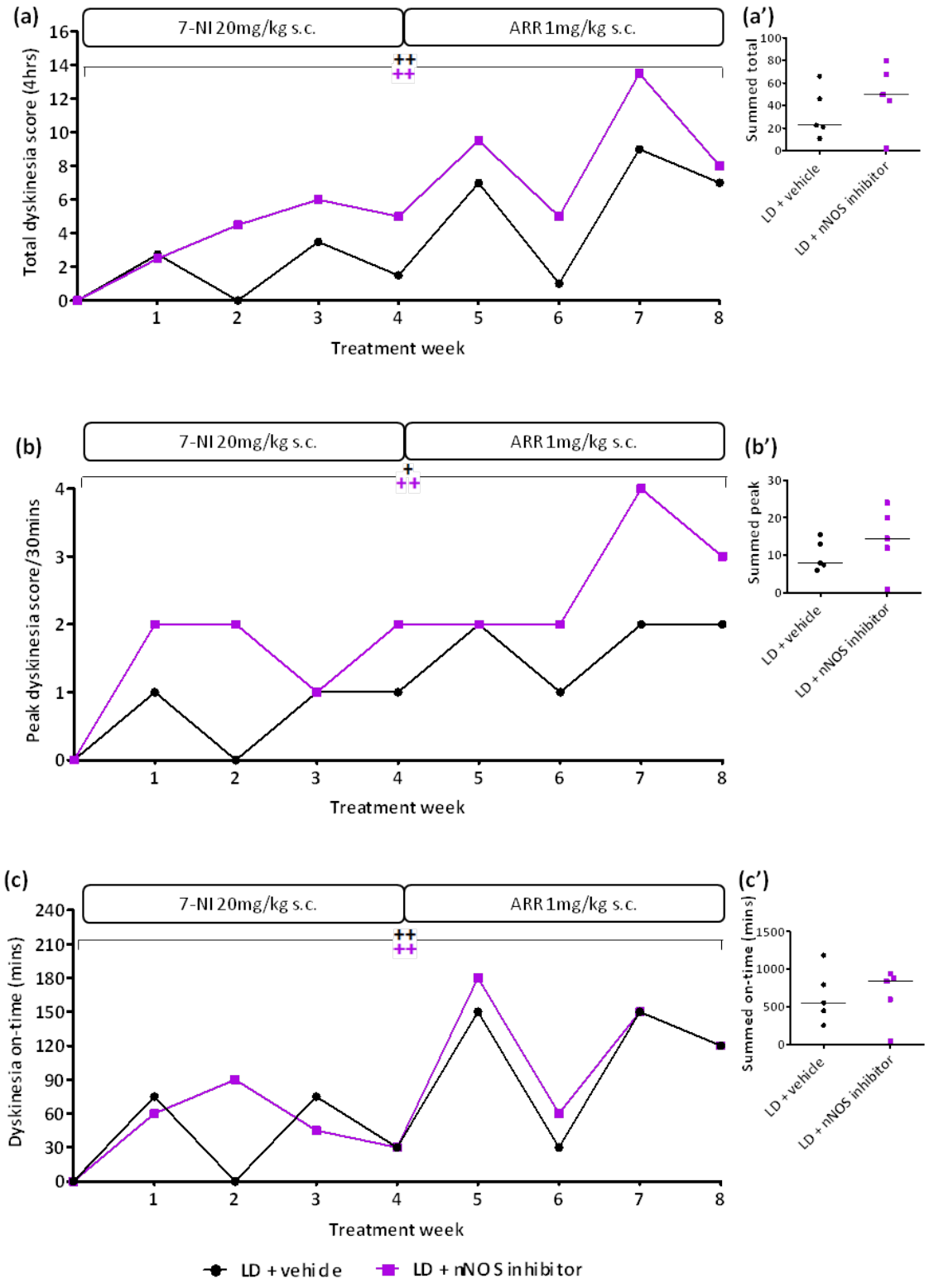

Figure 5-11 Dyskinesia following chronic treatment with nNOS inhibitor plus L-dopa. 7-NI (20 mg/kg s.c.; weeks 1-4) or ARR17477 (ARR; 1 mg/kg s.c; weeks 5-8) and L-dopa (LD; 12.5 mg/kg plus carbidopa 12.5 mg/kg p.o.) treatment in MPTP-treated marmosets, L-dopa naïve at week 0 (t=0 indicates baseline activity). Data are presented as medians (n=5); **(a)** Total, **(b)** Peak and **(c)** On-time, and also individual values **(a')** Summed total, **(b')** Summed peak and **(c')** Summed on-time; +p<0.05, ++p<0.01 for time (colours representative as in key). Weekly behavioural data were analysed by 2-way-ANOVA followed by Friedman's test for time. Summed data were analysed by Mann-Whitney tests.

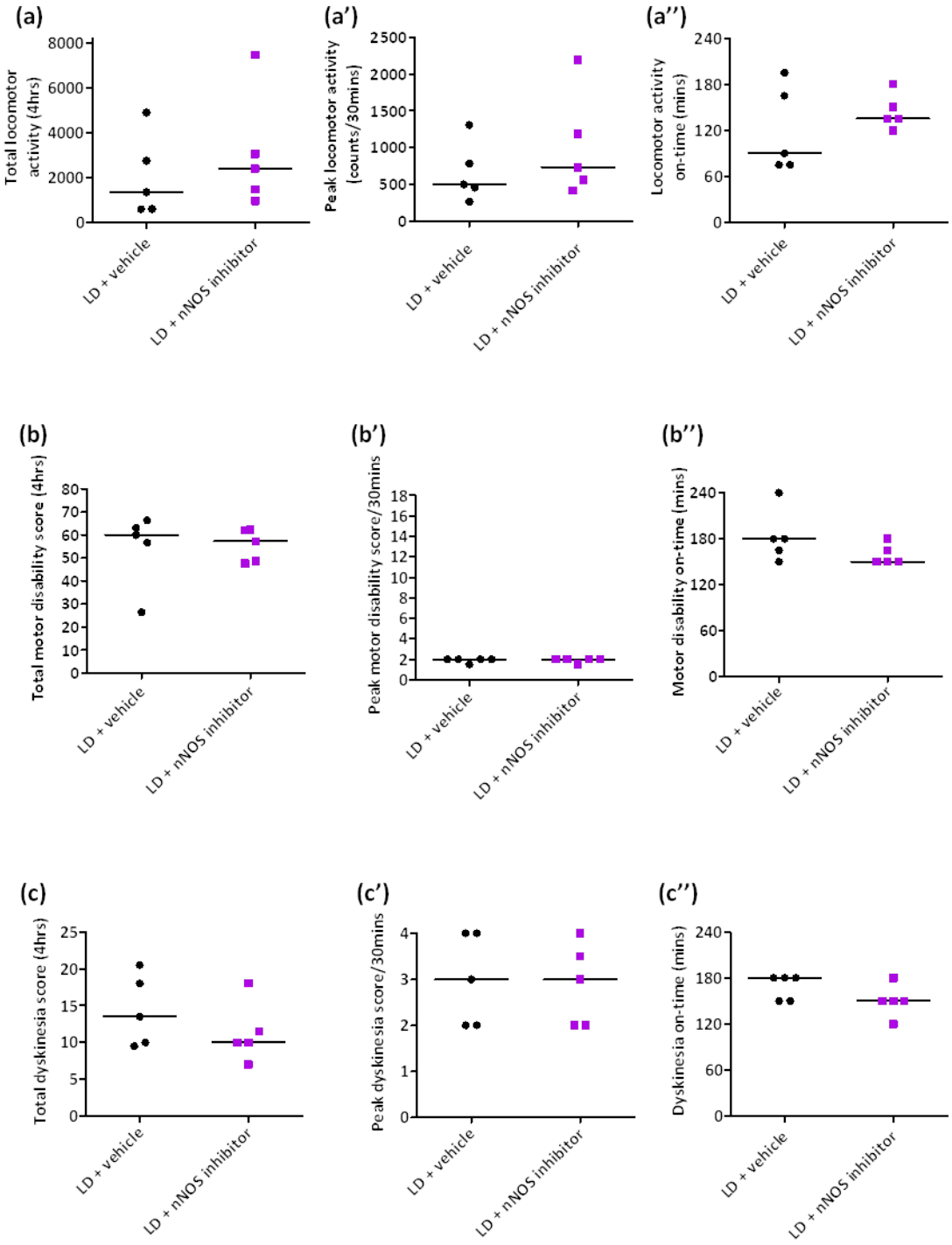

Figure 5-12 Final L-dopa only challenge data subsequent to chronic nNOS inhibitor plus L-dopa treatment. L-dopa (LD; 12.5 mg/kg plus carbidopa 12.5 mg/kg p.o.) treatment in L-dopa plus nNOS inhibitor/vehicle primed MPTP-treated marmosets. Data are presented as medians and individual values (n=5) and groups are labelled according to their prior chronic treatment. Locomotor activity **(a-a'')**, motor disability **(b-b'')** and dyskinesia **(c-c'')**; totals **(a-c)**, peaks **(a'-c')** and on-time **(a''-c'')**. Data were analysed by Mann-Whitney tests and is a mean of two final acute challenges.

5.4 Discussion

The studies described in this chapter explored the application of nNOS inhibitors in treatment of established dyskinesia and also in the process of dyskinesia development in MPTP-treated marmosets. It was hypothesised that nNOS inhibition would both reduce the expression of L-dopa- and ropinirole-induced dyskinesia in L-dopa-primed MPTP-treated primates, and also prevent the induction of dyskinesia from first L-dopa exposure.

Initially it was important to confirm that 7-NI promotes nNOS inhibition when administered via a s.c. route, as i.p. dosing is not approved in primates, whilst the doses of ARR17477 were chosen in line with previous rodent studies, where the treatment was already given s.c., and supported by the literature. These doses were then applied to *in vivo* investigations in MPTP-treated primates in a similar manner to those employed in 6-OHDA-lesioned rats in Chapters 3 and 4.

5.4.1 Inhibition of nNOS by ARR17477

In the dyskinesia expression studies, the doses of ARR17477 (3, 6 and 12 mg/kg s.c.) were selected on the basis of a previous study in rats showing nNOS activity was reduced in striatal tissue at all doses employed, in a dose-related manner and the highest two doses significantly reduced nNOS activity in cerebellar tissue (see Chapter 3, section 3.2.3). These findings were in agreement with published work on nNOS inhibition by ARR17477 (Zhang *et al.*, 1996; O'Neill *et al.*, 2000; Reif *et al.*, 2000), as discussed in Chapter 3. For the priming study involving chronic treatment with nNOS inhibitor, the dose of ARR17477 (1 mg/kg s.c.) was chosen as previously described, showing a significant reduction in nNOS activity at 4 days, taken to be indicative of cumulative chronic treatment, without any overt side-effects (see Chapter 4, section 4.2.3.2). It was expected that these doses would be similarly effective in primate as rodent. It was important that the dose of ARR17477 should reduce nNOS activity without affecting basal motor function as sometimes noted with NOS inhibitors (Sandi *et al.*, 1995; Dzoljic *et al.*, 1997) and by lowering the dose as far as possible for the chronic study, the chances of such an outcome would be minimised.

5.4.2 Inhibition of nNOS by 7-NI

The *ex vivo* study reported in this chapter showed that 7-NI (20 mg/kg s.c.) significantly reduced nNOS activity by 20 % in striatal tissue, although there was no significant effect in cerebellum. As the main site of interest for nNOS effects relating to motor output is the striatal interneurones where levels of nNOS expression per neuron are especially high (Bredt *et al.*, 1991; Nisbet *et al.*, 1994), the changes in striatal activity were considered most pertinent to these studies. Levels of inhibition were considerably lower following s.c. than i.p. administration of a comparable dose, whereby the former route reduced striatal nNOS activity by 41 % (See Chapter 3). This variation may be due to the fact that 7-NI was given as a suspension in this case. Measured at a later time point inhibition may have been higher, owing to continuous slow release of the nNOS inhibitor. It is also important to bear in mind that some enzyme-inhibitor dissociation is likely to have occurred especially considering the reversible nature of 7-NI so

true levels of inhibition may well be higher and therefore 20 mg/kg s.c. should be an effective dose to use. Differences are also likely to be applicable to *ex vivo* studies in previous chapters (Chapters 3 & 4), where although not commented upon at the time, in retrospect may have had higher levels of nNOS inhibition owing to enzyme-inhibitor dissociation than strictly indicated by the measured changes in enzyme activity. Hantraye *et al.* (1996) indeed demonstrated that a similar dose of 7-NI (25 mg/kg s.c.), administered in peanut oil, prevented MPTP-induced neurotoxicity in primates. Although no nNOS *ex vivo* data for this route were presented, it suggests that this dose was effective at inhibiting NOS and the authors claim the findings were not due to any 7-NI related MAOB effects.

5.4.3 The effect of acute nNOS inhibition on L-dopa-expressed behaviours in MPTP-treated marmosets

The highest dose of ARR17477 (12 mg/kg s.c.) had no significant effect on locomotor activity, motor disability or dyskinesia in MPTP-treated primates. These findings are in agreement with effects seen in 6-OHDA-lesioned rats also treated with ARR17477 alone (see Chapters 3 & 4), and in a study by Johansson *et al.* (1999) in rodents showing no change in locomotion or other gross behaviours following ARR17477 (0.5-20 mg/kg s.c.) administration.

L-dopa alone induced an increase in locomotor activity, reversal of motor disability and onset of dyskinesia as expected from preliminary experiments (see dose-response, chapter 2, section 2.5.5) and as previously reported (Clarke *et al.*, 1987; Pearce *et al.*, 1995). Dyskinesia expression coincided with alleviation of motor disability both lasting for an equal duration and hence analogous to 'peak-dose' dyskinesia where abnormal movements are maximal where L-dopa plasma concentrations peak as seen in PD patients (Schneider, 1989).

ARR17477 (3-12 mg/kg) treatment caused some potentiation of L-dopa-induced locomotor activity increasing total counts by 100 %, although statistically this did not prove significant compared to L-dopa alone likely to be due to due to the large variability evident in the animals' responses. L-dopa-induced reversal of motor disability and dyskinesia were not significantly affected by ARR17477 treatment.

Underlying this tendency for increasing locomotor activity may be non-nitergic effects of ARR17477, which has shown affinity to non-dopaminergic receptors, particularly α2, and the vesicular monoamine transporter (VMAT), dopamine transporter (DAT) and norepinephrine transporter (NET) in a ligand binding assay (See Appendix, Table 0-1). Although it is not possible to tell whether effects would be inhibitory or excitatory from these data, speculation on activity leading to an increased locomotor activity could be made. Inhibition of VMAT, involved in vesicular storage for regulation of sustained dopamine release, would be likely to increase locomotor activity as a result of higher levels of extracellular dopamine, and the inhibition of NET has been shown to enhance L-dopa-induced dopamine release which would be expected to have a similar effect (Liu & Edwards, 1997; Arai *et al.*, 2008). Additionally inhibition of DAT and antagonist activity at adrenergic α2 receptors have both been associated with increased extracellular dopamine and have been shown to promote locomotor activity

(Grondin *et al.*, 2000; Madras *et al.*, 2006). Inhibitory/antagonist activity of ARR17477 at these receptors or transporters could therefore potentially elevate locomotor activity. The activity is unlikely to be directly related to nNOS inhibitory properties of ARR17477 as NOS inhibitors would be expected to have the opposite effect having been shown to reduce spontaneous locomotion in animals (Stewart *et al.*, 1994; Sandi *et al.*, 1995).

Interestingly there was no significant effect or definitive trend for ARR17477 on L-dopa-induced reversal of motor disability suggesting it does not interfere with the antiparkinsonian action of L-dopa. One might expect an increase in motor disability reversal if extracellular dopamine levels are potentiated as suggested above, however motor disability scores are assessed using a finite scale and rarely exceed a peak score of 2, as most drugs do not completely normalise vocalisation in animals.. By contrast locomotor activity counts are continuous data with the potential to increase infinitely, hence there is no ceiling effect constraining measurement, which is likely to explain the apparent lack of further change seen in motor disability.

According to the present results, nNOS inhibition does not reduce L-dopa expression of dyskinesia in the MPTP-treated primate. Meanwhile, various studies show that L-dopa-induced dyskinesia expression can indeed be reduced in this model. Amantadine, a weak NMDA receptor antagonist used in the clinic reduces dyskinesia (Blanchet *et al.*, 1998a; Hill *et al.*, 2004). Similarly other NMDA antagonists have been shown to alleviate dyskinesia in this model including LY235959 and the NR1A/NR2B subtype-specific antagonist Co 101244 (Papa & Chase, 1996; Blanchet *et al.*, 1999). AMPA receptor antagonists topiramate and LY300164 (Konitsiotis *et al.*, 2000; Silverdale *et al.*, 2005) and metabotropic glutamate receptor type 5 (mGluR5) antagonists MTEP and MPEP (Johnston *et al.*, 2010; Morin *et al.*, 2010) also reduce dyskinesia in MPTP-treated primates, highlighting the important role of glutamate in the expression of abnormal motor effects.

Modulation of other neurotransmitter pathways has also generated successful results with 5-HT1a agonists Sarizotan and (+)-8-OHDPAT reducing L-dopa-induced dyskinesia although the latter drug compromised reversal of motor disability Iravani *et al.*, 2006b) (Bibbiani *et al.*, 2001; Iravani *et al.*, 2006). Combination of 5-HT1a and 5-HT1b agonists has also decreased L-dopa-induced dyskinesia in primates, as also seen with MDMA believed to act on these receptors (Iravani *et al.*, 2003; Munoz *et al.*, 2008). Quetiapine, having 5-HT2A/C and D2/3 antagonistic activity reduces expression of L-dopa-induced dyskinesia (Oh *et al.*, 2002), whilst a partial D_3 receptor agonist has similar effects (Bezard *et al.*, 2003), hence the specific receptor sub-type targeted seems pertinent. The α2 adrenoreceptor antagonists idazoxan and fipamezole have also demonstrated anti-dyskinetic efficacy in this model (Henry *et al.*, 1999; Savola *et al.*, 2003), although both of these compounds only delayed the onset of dyskinesia without reducing its overall expression in studies conducted in our laboratories (M. Jackson, unpublished data). Further targets for dyskinesia attenuation in MPTP-treated primates have included cannabinoid receptor agonists (Fox *et al.*, 2002), opioid μ- and δ-receptor antagonists (Henry *et al.*, 2001) and adenosine A2a receptor antagonists in some cases (Kanda *et al.*, 2000). Notably some of

these dyskinesia-modifying drugs are relatively non-specific so it is difficult to know the exact mode of action or benefits may be due to multiple effects. However, the wide array of L-dopa adjuncts able to moderate motor complications demonstrate that it is certainly possible to reduce the expression of dyskinesia in MPTP-treated primates and hence suggests nNOS inhibitors are inappropriate agents for this purpose.

Importantly, this study confirms the findings seen in the 6-OHDA-lesioned rat (Chapter 3) showing that nNOS is not critically involved in the expression of L-dopa-induced dyskinesia regardless of the model employed.

5.4.4 The effect of acute nNOS inhibition on ropinirole-induced behaviour in MPTP-treated primates

Treatment with ropinirole caused an increase in locomotor activity, reversal of motor disability and onset of dyskinesia in L-dopa-primed MPTP-treated marmosets as seen in preliminary experiments (See dose-response, chapter 2). As expected, ropinirole resulted in lower levels of dyskinesia expression than produced by L-dopa. This contrast reflects published findings comparing the dyskinetic potential of ropinirole and L-dopa both in primates and 6-OHDA-lesioned rats (Jackson *et al.*, 2007; Papathanou *et al.*, 2011), and also earlier studies presented in this thesis (see Chapter 3, section 3.4.3). Whilst ropinirole is short-acting in rats, this study confirms that in primates it is longer acting, with effects on behavioural parameters lasting for a comparable time to L-dopa and beyond (Pearce *et al.*, 1998; Fukuzaki *et al.*, 2000b). This observed difference lends support to the similarity between drug responses in PD patients and the MPTP-treated primate, where ropinirole is also relatively long-acting. Meanwhile the extent of motor disability reversal was similar to that observed following L-dopa only treatment, although ropinirole appeared to induce higher total locomotor activity levels than L-dopa alone, despite an equivalent duration of locomotor activity. According to previous studies, locomotor activity levels in primed MPTP-treated marmosets tend to be comparable between L-dopa and ropinirole (Pearce *et al.*, 1998). Thus the difference found in the present study is likely to be attributable to the on-going low level activity, observable at 3-5 h after ropinirole dosing, only marginally above baseline levels which may not have counted towards median 'on-time' but would have contributed to totals for locomotor activity, owing to the nature of the different parameters.

There was no significant effect of ARR17477 on ropinirole-induced locomotor activity, reversal of motor disability or dyskinesia. It is not surprising that ARR17477 did not show a similar trend for increasing locomotor activity as seen when administered in combination with L-dopa. As a dopamine agonist ropinirole's effects are predominantly post-synaptic and therefore the non-nitrergic activity of the nNOS inhibitor, which would mainly affect pre-synaptic neurone terminals (as described in 5.4.3), is unlikely to interfere in this case.

Although there are limited studies on the effects of adjuncts on ropinirole-induced dyskinesia expression, experiments demonstrate that motor abnormalities following dopamine agonist treatment

can be attenuated in the MPTP-treated primate. For example MDMA in combination with pramipexole, also a D_2/D_3 agonist, reduces the expression of dyskinesia and the moderation is likely to be due to interactions with 5-HT pathways (Iravani *et al.*, 2003). This mechanism was further supported by work with (+)-8-OHDPAT, a selective agonist at 5-HT1a receptors, which also reduced dyskinesia expressed by pramipexole although only with a reduction in motor disability reversal (Iravani *et al.*, 2006). Additionally morphine, an opioid agonist, attenuates dyskinesia expressed by the D_2 receptor agonist, quinpirole (Samadi *et al.*, 2004). Therefore the expression of dyskinesia by dopamine agonists with similar D_2 affinity to ropinirole can be successfully reduced in the MPTP-treated primate model. The inability for ARR17477 to reduce dyskinesia expression by ropinirole undermines the use of nNOS inhibitors for treatment of motor complications in this model, and also the chances of benefit in man given the translational link between species (Fox *et al.*, 2006; Porras *et al.*, 2012).

These findings further support the outcome seen for ARR17477 on L-dopa expressed dyskinesia. Again, the ineffectiveness of ARR17447 in this study confirms the results from the 6-OHDA-lesioned rat model of Chapter 3, and a lack of involvement of nNOS in ropinirole-induced dyskinesia expression.

5.4.5 The effect of chronic nNOS inhibition on L-dopa-induced behaviour in MPTP-treated primates

The second set of experiments in this study investigated the effect of nNOS inhibition in the priming for dyskinesia by L-dopa. Priming could present an earlier opportunity to impede upon alterations in synaptic plasticity resulting from changes in nitric oxide, which may underlie the initial development of dyskinesia, and for which manipulating at the expression stage (sections 5.4.3 and 5.4.4) may have been too late on to confer benefit.

Chronic administration of L-dopa raised locomotor activity, reversed motor disability and gradually induced dyskinesia over the treatment period. These findings were consistent with the effect of L-dopa reported in previous priming studies in MPTP-treated primates (Bedard *et al.*, 1986; Boyce *et al.*, 1990; Pearce *et al.*, 1998; Smith *et al.*, 2002). As expected, the reversal of motor disability and increase in locomotor activity was seen immediately after initiating treatment, although the duration of improved motor function tended to increase over the chronic treatment period. The onset of dyskinesia was more gradual with maximum scores not achieved until week 5. This is somewhat different to the onset of dyskinesia in rats which is almost immediate with maximal AIMs scores tending to be reached within the first week (See Chapter 4). However it does not completely reflect the delay in dyskinesia expression in man, where many years of dyskinesia free dopaminergic therapy are commonly experienced (Fahn, 2000; Rascol *et al.*, 2000; Ahlskog & Muenter, 2001). In primate and rodent models, in a similar manner to patients with PD, the severity of dyskinesia relates to the degree of parkinsonism and the dose and duration of levodopa therapy (Schneider *et al.*, 2003; Smith *et al.*, 2003). Both MPTP-treated primates and 6-OHDA-lesioned rats have a severe depletion of dopaminergic neurones most similar to the

neuropathology evident in advanced stages of PD, which explains why dyskinesia is induced so rapidly in these models.

Similar changes in motor behaviour were seen with L-dopa administered in combination with nNOS inhibitor compared to L-dopa alone. There was no difference in locomotor activity, reversal of motor disability or indeed dyskinesia between the two treatment regimes. Considering the lack of effect of nNOS inhibitors seen in 6-OHDA-lesioned rats the slower onset of dyskinesia in this model seemed more likely to allow for preventative therapy, but this difference in timing did not appear to alter the outcome. Furthermore after drug washout final acute challenges with L-dopa alone in both groups showed no effect of nNOS inhibitor on the priming process.

It became necessary to change nNOS inhibitor during the course of chronic treatment due to skin lesions developing at 7-NI injection sites in several animals. 7-NI has poor solubility in polar solutions so it was not possible to use saline as vehicle and we had already anticipated that DMSO, as used in the rat studies involving 7-NI (Chapters 3 & 4), would cause skin problems. The decision was made to switch to ARR17477 which was the original choice of nNOS inhibitor owing to its more favourable properties of nNOS selectivity and potency, but was not employed initially in this primate model due to concerns over chronic implications of its long acting and irreversible nature. The switch occurred from one day to the next so it was ensured nNOS inhibition was consistent with subsequent L-dopa dosing.

Although nNOS activity in rat striatum was shown to be reduced by subcutaneous administration of 7-NI in this chapter, there was no effect seen in the cerebellum. Whilst the striatum is considered the main site of interest for nNOS as discussed in section 5.4.2, the cerebellum is also known to be important in movement control and may contribute to the motor complications seen in PD (Hurley *et al.*, 2003; Koch *et al.*, 2009). Therefore it cannot be ruled out that 7-NI may have failed to lessen the development of dyskinesia owing to the fact it did not reduce cerebellar nNOS activity. Nevertheless when ARR17477 treatment, already demonstrated to reduce nNOS activity in rat cerebellum (see Chapter 4), was introduced in week 4 dyskinesia levels were sub-maximal and there did not appear to be any prevention of dyskinesia progression in subsequent weeks. Potentially it could have been too late to resist underlying molecular changes by this stage of dopaminergic treatment. Notably it is also possible that the extent of nNOS inhibition may have differed between marmosets and rats in any case, but primates could not be spared for sacrifice and this is a shortcoming of the present study.

Contrary to the predicted reduction in dyskinesia priming by nNOS inhibition, there was a trend towards an increase in dyskinesia over the full priming period. Based on the rat priming data (Chapter 4) where a similar effect was seen during L-dopa priming with 7-NI on AIMs, and indeed statistical significance was achieved, it seems plausible that again this increase may be due to the MAOB inhibitory properties of 7-NI (Castagnoli *et al.*, 1997; Di Monte *et al.*, 1997; Desvignes *et al.*, 1999) in addition to possible eNOS effects as discussed previously. Once dyskinesia had been induced the required switch of nNOS inhibitor to ARR17477 (with less evidence for MAOB inhibition-see Chapter 3, section 3.4.2) may have occured too late on to modify molecular changes which had already taken place associated with priming.

This is the first time that nNOS inhibition has been investigated in the priming of MPTP-treated primates. There are a number of other agents that have been used as adjuncts to L-dopa successfully lessening the development of dyskinesia including the COMT inhibitor entacapone (Smith *et al.*, 2005), the mGluR5 antagonist fenobam (Rylander *et al.*, 2010), CI-1041, a selective antagonist of the NR1A/2B subtype of NMDA receptor (Hadj Tahar *et al.*, 2004; Samadi *et al.*, 2008) and D3 antagonist S33084 (Visanji *et al.*, 2009). These studies demonstrate the potential for manipulation of the priming process in this model and the capability to reduce the development of dyskinesia. In particular these reported studies support the modulation of glutamate for attenuating priming for dyskinesia, thus suggesting that nNOS is not of key involvement despite its implicated role downstream of glutamatergic changes. Indeed antagonism at the glutamate receptors themselves (mGluR5 or NMDA NR1A/2B) appears to be critical, perhaps because it can lead to broader effects than solely altering levels of NOS which the present studies have revealed is insufficient.

This priming study shows that nNOS is not a crucial factor in the development of L-dopa-induced dyskinesia in MPTP-treated primates.

5.4.6 Conclusion

The data presented here from MPTP-treated primates confirm the findings obtained in 6-OHDA-lesioned rats of previous chapters. It leads to a conclusion that nNOS inhibition is ineffective in reducing expression and priming for dyskinesia in these animal models. Given the likeness of MPTP-treated primate motor dysfunction and dyskinesia to associated behavioural manifestations in PD, coupled with studies which have in fact shown that expression and priming for dyskinesia can both be attenuated in this translational model, these findings have strong implications. Thus in conclusion, it is unlikely that nNOS inhibitors would provide any relevant benefit for dopaminergic medication-induced dyskinesia in a clinical setting.

Chapter 6 : General discussion

6.1 Summary and discussion of findings

It was hypothesised that NO produced by nNOS is critically involved in the occurrence and evolution of dyskinesia in PD, via synaptic plasticity mechanisms, and this can be controlled by inhibition of nNOS. Thus these studies investigated whether nNOS inhibition could reduce the expression of dyskinesia in animals already treated long-term with dopaminergic agents and also the priming for dyskinesia from first exposure of animals to dopaminergic treatment. In order to explore these phenomena two animal models of PD were employed, both of which display abnormal involuntary movements following dopaminergic treatment; the 6-OHDA-lesioned rat and the MPTP-treated marmoset.

6.1.1 Dyskinesia expression and NO

In 6-OHDA-lesioned rats already L-dopa-primed to show AIMs, the rodent form of dyskinesia, the inhibition of nNOS had no beneficial effect on L-dopa- or ropinirole-induced AIMs expression. In fact some potentiation of total AIMs scores and duration of AIMs was evident following 7-NI treatment, although this effect may have been the result of inhibition of MAOB and/or eNOS. Similarly in L-dopa-primed MPTP-treated marmosets, the expression of dyskinesia was unaltered by the inhibition of nNOS in combination with L-dopa or ropinirole treatment, whilst locomotor activity and motor disability reversal increased in the same manner as for dopaminergic treatment alone.

6.1.2 Dyskinesia priming and NO

The inhibition of nNOS during *de novo* L-dopa treatment in 6-OHDA-lesioned rats did not prevent the development of AIMs. Indeed, as seen in the expression studies, AIMs were worsened following 7-NI treatment, most likely the result of inhibition of MAOB and/or eNOS. Whilst there was some tendency for ARR17477 to reduce the development of AIMs, no statistical significance was achieved. These findings were corroborated by the L-dopa priming study in MPTP-treated marmosets showing that the inhibition of nNOS does not influence the development of dyskinesia, whilst locomotor activity and motor disability reversal over the priming period were also unaffected.

This is the first time that the role of nNOS inhibitors has been investigated in the expression and priming of dyskinesia in both rodent and primate models of PD. Importantly, the accompanying *ex vivo* studies thoroughly verify the inhibition of nNOS by ARR17477 and 7-NI at the doses employed in the behavioural investigations, allowing confidence in concluding that nNOS does not play a significant role in these phenomena. These findings lead to a rejection of the proposed hypothesis that NO underlies the motor complications and indeed suggest that the inhibition of nNOS is unlikely to be of any value in the treatment of dyskinesia in the clinic. Interestingly these findings are in contrast to those described in the literature, published whilst the present work was undertaken (Padovan-Neto *et al.*, 2009; Takuma *et al.*, 2012), and it is important to consider why this may be the case.

Padovan-Neto *et al.* (2009), reported a reduction in expression of dyskinesia by nNOS inhibiton in 6-OHDA-lesioned rats and more recent research by Takuma *et al.* (2012), only in publication at the conclusion of writing this thesis, reported attenuation of the development of dyskinesia by nNOS inhibition also in this rat model. Both studies have significant drawbacks, firstly their use of only two time points for AIMs assessment at 60 and 120 min after dopaminergic treatment which may well obscure any changes occurring at other times. This monitoring period is not in keeping with the established methods of AIMs evaluation in 20 min cycles as developed by Cenci *et al.* (1998) and widely employed across AIMs studies (e.g. Monville *et al.*, 2005; Pinna *et al.*, 2006; Putterman *et al.*, 2007; Marin *et al.*, 2009). In fact the experiments carried out in the present studies measured AIMs every 15 min rather than 20 min, so are yet more stringent in portraying the ongoing changes in behaviour over the full time-course of analysis.

Secondly the suitability of the doses of drugs employed in the published studies was questionable. Extraordinarily high doses of L-dopa (up to 30-100 mg/kg) were utilised by both groups suggesting that locomotive behaviour would obscure the capacity to score AIMs, as already discussed in Chapter 3 (see section 3.4.2). Higher doses of L-dopa are known to promote dyskinesia, but are rarely employed in PD patients these days making these published studies less clinically relevant. Additionally, in neither investigations were *ex vivo* experiments reported to validate their chosen doses of 7-NI. In particular Takuma *et al.* (2012) used only 7-NI 10 mg/kg which is low compared to the typical dose of 25-30 mg/kg used for priming in Chapter 4 and commonly reported in the literature (Mackenzie *et al.*, 1995; Jiang *et al.*, 2002; de la Torre & Aliev, 2005; Wangensteen *et al.*, 2006). Additionally according to the *ex vivo* data shown in Chapter 3 whilst their dose is likely to cause some inhibition of nNOS it may not be optimal, although it is possible at this lower dose that non nNOS-specific effects are indeed minimised. In neither study is there any hint of 7-NI increasing AIMs as seen in both the expression and priming experiments as reported in this thesis, despite Padovan-Neto and colleagues using 7-NI 30 mg/kg. Moreover the only nNOS-specific inhibitor employed in both of these studies was 7-NI, whereas the experiments reported here additionally test ARR17477 which is more potent and indeed specific for nNOS over other NOS isoforms (see section 6.1.3).

Constructively, Takuma and colleagues (2012) carried out some biochemical investigations to accompany their behavioural findings showing that 7-NI lessened the L-dopa-induced increases in ΔFosB and phosphorylated DARPP-32 in the 6-OHDA-lesioned striatum. As no significant benefit was conferred by nNOS inhibitors in the presented studies there was no rationale for investigating the biochemistry any further.

Importantly the present studies used well established protocols in both rodent (Cenci *et al.*, 1998; Winkler *et al.*, 2002; Putterman *et al.*, 2007) and marmoset models (Pearce *et al.*, 1995; Silverdale *et al.*, 2005; Jackson *et al.*, 2007), which have been fully characterised to show a reduction in dyskinesia with established drugs including amantadine. If nNOS inhibition were to be clinically relevant then reductions

in AIMs/dyskinesia as seen with such drugs would have been observed in both models but this was not the case.

6.1.3 Specificity of NOS inhibitors

The availability of specific CNS penetrant inhibitors for *in vivo* assessment of the role of inhibition of nNOS on dyskinesia was somewhat limited. In these studies ARR17477 was chosen as the best available tool for this purpose, in addition to 7-NI which has been employed in many studies for nNOS inhibition (e.g. Przedborski *et al.*, 1996; Li *et al.*, 2002; Wangensteen *et al.*, 2006). Compared to similar drugs ARR17477 in particular is reportedly relatively specific for nNOS over other NOS isoforms (Vallance & Leiper, 2002; Paige & Jaffrey, 2007). Additionally results from the *ex vivo* studies of Chapters 3-5 strongly support the capacity for NOS inhibition by ARR17477 and 7NI using the chosen doses and routes of administration.

The increased duration of AIMs following 7-NI is consistent with the reported inhibitory effect on MAOB and/or eNOS inhibitory activity as already discussed in Chapters 3 and 4. Indeed, the notable trend for ARR17477 to attenuate AIMs suggesting a possible antidyskinetic function that was masked by other effects of the drug, possibly through binding to DAT, VMAT, NET or adrenergic α2 receptors as identified in a ligand binding assay (See Appendix; Table 0-1). Although the excitatory/inhibitory nature of the interaction cannot be deduced there is evidence for a role for these transporters and receptors in worsening dyskinesia (Fox *et al.*, 2001; Nutt *et al.*, 2004; Lee *et al.*, 2006; Arai *et al.*, 2008).

Ideally the nNOS inhibitors would have been more specific and the differences in their behavioural effects on dyskinesia in some instances highlight this (worsening of dyskinesia evident with 7-NI but not with ARR17477 particularly suggests 7-NI is by no means a perfect probe for investigating nNOS specific effects). It was therefore important to consider the effects of two different inhibitors thus adding further weight to the present findings in comparison to those of Padovan-Neto *et al.* (2009) and Takuma *et al.* (2012), whereby 7-NI was the only nNOS inhibitor tested.

Several groups are working on producing more specific inhibitors of nNOS and provided these prove effective *in vivo* a more valuable tool set is likely to be available for investigations in the future (Joubert & Malan, 2011; Annedi *et al.*, 2012; Ramnauth *et al.*, 2012). Additionally the direct intracerebral delivery of nNOS inhibitors could provide an approach to overcome reported issues of blood-brain barrier penetration associated with more recently synthesized nNOS-specific compounds (Xue *et al.*, 2010a).

Notably the most consistent finding overall in these studies was that neither of the nNOS inhibitors significantly reduced dyskinesia in the 6-OHDA-lesioned rat or MPTP-treated primate at doses shown to reduce nNOS activity. These results therefore show that nNOS inhibition is an ineffective mechanism for reducing dyskinesia.

6.1.4 The animal models

6.1.4.1 6-OHDA-lesioned rat

6-OHDA-lesioned rats are the most commonly used animal model for investigating motor complications of PD treatment, as they are both accessible and readily demonstrate quantifiable behaviours (Cenci & Ohlin, 2009). The pharmacological profiling of the AIMs model employed in these studies support validation of AIMs within the literature. The characterisation experiments presented in Chapter 2 showing amantadine, MK-801 and 8-OHDPAT all reduce AIMs are in agreement with reported studies (Dekundy *et al.*, 2007; Dupre *et al.*, 2008). Additionally the dopaminergic AIMs expression and priming studies demonstrated in Chapters 3 and 4 with only L-dopa or ropinirole treatment in 6-OHDA-lesioned rats further support findings in the literature (e.g. Carta *et al.*, 2008a; Monville *et al.*, 2009; Papathanou *et al.*, 2011). These confirm the reliability and reproducibility of the model for displaying AIMs and its validity within the context of experiments presented in this thesis.

AIMs in 6-OHDA-lesioned rats consist of locomotive, axial, limb and orolingual parameters (Cenci *et al.*, 1998; Lundblad *et al.*, 2002). The significance of locomotive AIMs as a measure of dyskinesia is uncertain and although originally included in the classification of the model (Cenci *et al.*, 1998), it has since been described as a 'non-specific' motor response (Winkler *et al.*, 2002). This may be true to a certain extent in that its exact cause is ambiguous although the response does seem specifically related to dopaminergic drug treatment. Rotational behaviour in 6-OHDA-lesioned rats was traditionally used to assess anti-parkinsonian activity, although sensitisation that develops following chronic dopaminergic treatment has been used as a model of priming for dyskinesia (Ungerstedt & Arbuthnott, 1970; Henry *et al.*, 1998). However, there exist several important differences between the manifestation of rotational behaviour and dyskinesia as measured by ALO AIMs: a) The levodopa dose necessary to induce stable rotational behaviour is higher than that for ALO AIMs expression, b) dopamine agonists with proven low-dyskinesiogenic potential induce a full rotational response and c) where 6-OHDA lesions are restricted to the dorsal striatum L-dopa induces ALO AIMs without rotational activity (Marin *et al.*, 2006; Cenci & Ohlin, 2009).

In the studies presented here locomotive AIMs tended to follow a similar pattern to ALO AIMs with both parameters showing significant changes or trends following drug treatments. However, locomotive AIMs have been considered independently in line with the literature where they are often omitted in more recent papers, or at least considered separately from the other three AIMs subtypes, and it is generally accepted that these are not representative of dyskinesia (Taylor *et al.*, 2005; Dupre *et al.*, 2007; Lindgren *et al.*, 2010). Furthermore, the essence of locomotive AIMs is undoubtedly different from the other AIMs subcategories which appear more analogous to the choreic and/or dystonic movements characteristic of dyskinesia in PD patients.

Similar to the clinical scenario AIMs are expressed following dopaminergic treatment and can be attenuated by various adjuncts, some of which are also beneficial in man. However there are increasingly more examples of drugs which show promise pre-clinically in this model but do not

translate effectively to humans such as the 5-HT1a agonist sarizotan and the α2 adrenoceptor antagonist idazoxan (Marin *et al.*, 2009; Buck *et al.*, 2010). One explanation for failure of translation may be down to limitations of the model itself. Extreme levels of dopaminergic depletion occur rapidly following administration of 6-OHDA almost incomparable to the slow progressive nature of degeneration characteristic of PD thus creating a model most reminiscent of end-stage PD. Additionally effects of the toxin tend to be restricted to dopaminergic neurones whereas pathology is more wide-spread affecting multiple neural pathways in PD. The extent of the damage is controlled by the quantity of toxin injected and the number and location of the injection sites, but typically the damage is severe and highly selective to the dopamine system. Indeed extensive neurodegeneration is necessary for 6-OHDA-lesioned rats to develop AIMs (Cenci, 2007). Furthermore the model centres on peak-dose dyskinesia, without displaying any 'on-off' phenomena or 'end of dose deterioration' as is seen in the clinic or indeed demonstrated in MPTP-treated primates (Langston *et al.*, 2000; Kuoppamaki *et al.*, 2002).

Whilst this model is not entirely predictive of clinical outcome, it does however demonstrate a reduction in L-dopa-induced dyskinesia with amantadine, the one agent that affords some proven benefit in PD patients (Luginger *et al.*, 2000; Lundblad *et al.*, 2002). Therefore whilst keeping its limitations in mind it can still provide a beneficial tool for investigating dyskinesia, especially where experimental outcome is considered alongside findings from another model of PD such as the MPTP-treated primate..

6.1.4.2 MPTP-treated marmoset

The MPTP-treated primate offers some clear advantages for assessing dyskinesia compared to the 6-OHDA rat AIMs model, not least the strikingly human-like choreic and dystonic movements they are capable of displaying upon dopaminergic medication affording strong face validity. Despite these benefits, the model also suffers many of the same mechanistic problems that have been associated with the 6-OHDA-lesioned rat.

Whilst dyskinesia induced by dopaminergic agents tend to develop a little more slowly from first drug exposure in MPTP-treated primates compared to 6-OHDA-lesioned rats, it still manifests within a very short period as compared to humans (weeks compared to years). Again, the rapid onset of dyskinesia is likely to be a reflection of the extensive degree of nigral denervation following toxin treatment, which lowers the threshold for dyskinesia induction. The situation in this primate model is obviously akin to the rapid appearance of treatment complications, including dyskinesia, in MPTP-exposed parkinsonian drug addicts (Langston *et al.*, 1983). It also more closely resembles the outcome seen in young-onset PD patients or in untreated late stage PD patients where dyskinesia promptly develop after initiating dopaminergic medication (Fabbrini *et al.*, 2007; Jenner, 2008b). The model may therefore have limited applicability to early stages of PD, where surviving nigrostriatal presynaptic terminals maintain a capacity to 'buffer' dopamine and hence striatal concentrations of dopamine do not show dramatic changes evident in later stages following continued neurodegeneration (Nutt, 2008). Indeed in the early

stage of disease dyskinesia development and expression is now relatively well controlled by titrating dopaminergic therapy, keeping doses low and plasma levels constant.

If a lower dose of L-dopa were to have been used this may have slowed down the rate of onset of dyskinesia (Kuoppamaki *et al.*, 2007). Similarly, it would be expected that dyskinesia induction and/or expression may be less severe with limited nigral denervation. In an ideal model the process of neurodegeneration would be more progressive, thus better resembling the course of events seen in man, and providing a wider window for development of dyskinesia. Indeed, the MPTP treatment regimen has been modified in some instances to induce partial lesions of the nigro-striatal pathway (Blanchet *et al.*, 1998b; Meissner *et al.*, 2003; Iravani *et al.*, 2005). However, L-dopa-induced dyskinesia is not demonstrated in the partially lesioned marmoset model, although some dyskinesia is seen in the partially lesioned squirrel monkey without overt symptoms of PD. This discrepancy may be due to differences in the number of L-dopa treatments given (Di Monte *et al.*, 2000; Iravani *et al.*, 2005).

Another significant drawback of the MPTP-model, is the lack of resemblance of pathology in noradrenergic and serotinergic neurons as seen in PD. As the MPTP-treated primate tends to act as a bridge between pre-clinical rodent studies and clinical investigations, it is even more significant at this stage that the model can distinguish between treatments likely to be effective in reducing dyskinesia and those that aren't. Whilst the MPTP-treated primate has been valuable in translation of dopaminergic agents to the clinic including adjuncts such as entacapone and dopamine agonists (Smith *et al.*, 1997; Fariello, 1998), the model has not shared similar success in progressing non-dopaminergic approaches to therapy. Failures including glutamatergic, noradrenergic, adenosinergic and serotonergic, acting drugs have occurred showing no translational benefit or an antidyskinetic action accompanied by worsened motor control in human trials (Fox *et al.*, 2006; Jenner, 2009).

In general behavioural data tend to show wide variability as has been consistently demonstrated throughout this thesis (see Chapters 3-5), for both rat and primate studies. In one respect this finding mimics more closely the clinical scenario where there are frequently large differences in drug response between individuals, however, it also can make it necessary to use very large sample sizes to achieve significant power. In terms of ethical concerns, practicality and costs it tends not to be possible to employ the number of animals designated by power analysis especially in the case of primates. The quantities of animals used in these studies are similar to those commonly reported in the literature by different groups where 8-10 6-OHDA-lesioned rats is typical of AIMs studies (Taylor *et al.*, 2005; Dekundy *et al.*, 2007; Putterman *et al.*, 2007; Monville *et al.*, 2009), and 5-6 MPTP-treated primates is usual for the purpose of investigating dyskinesia (Pearce *et al.*, 1998; Blanchet *et al.*, 1999; Henry *et al.*, 2001; Silverdale *et al.*, 2004; Gregoire *et al.*, 2009).

Ideally a perfect animal model would mimic all aspects of the disease with respect to the symptoms and underlying biochemical changes, and these should occur over a similar time frame to those seen in man.

Whilst this imitation may be possible to some extent with genetic models these only tend to show pathological changes in specific pathways and so far seem not to display dyskinesia. Meanwhile, both the 6-OHDA-lesioned rat and MPTP-treated primate have shown the capacity for dyskinesia to be reduced by adjunctive agents in dyskinesia expression and priming studies with dopaminergic drugs as described in individual chapter discussions (See Chapters 3-5). Therefore, despite their shortcomings, they are useful tools for investigating dyskinesia pre-clinically and the lack of effect seen with nNOS inhibition is highly unlikely to be due to inherent faults of the models employed. These qualities further validate the conclusion reached from experiments in these models, that nNOS inhibition does not reduce the expression or priming of dyskinesia.

6.2 Is there a role for glutamate derived NO in dyskinesia?

Despite robust pre-clinical and clinical evidence for a key role of NO in PD and dyskinesia expression and/or priming, the current results imply that NO is not critical and indeed attenuation of NO levels confers no obvious benefit for addressing motor complications. Whilst the studies of Padovan-Neto *et al.* (2009) and Takuma *et al.* (2012), employing 7-NI for reduction of AIMs in 6-OHDA-lesioned rat suggest otherwise, as discussed fully in section 6.2.1, the more rigorous and clinically-relevant investigations presented in this thesis lead to an overriding conclusion that NO is not fundamental in dyskinesia expression or priming.

NO is known to interact with multiple neurotransmitter pathways and so reducing NOS activity by nNOS inhibition is unlikely to exclusively reduce NO production associated with glutamatergic activity and synaptic plasticity. It would also be expected to influence other neurotransmitters including serotonin, adenosine, noradrenaline and dopamine (Prast & Philippu, 2001), all of which could further impact upon dyskinesia. Furthermore, moderating NO effects may not be achievable to the same degree as for conventional neurotransmitters e.g. 5-HT, where receptors tend to be specifically associated with neuronal membranes and interactions highly localised. NO acts as an atypical neurotransmitter/neuromodulator not constrained by cellular membranes and can freely diffuse through aqueous and lipid environments in three dimensions, yet adding further complexity to predicting its CNS effects.

The regulation of molecular events may also be required further upstream from NO release as additional changes resulting from overactive (NMDA) glutamate levels as well as increased NO may significantly contribute towards dyskinesia. For example, selective antagonism of the NR1A/2B subtype of NMDA receptor has shown promise in alleviating the expression and development of dyskinesia in animal models although translation to the clinic is yet to prove successful (Blanchet *et al.*, 1999; Samadi *et al.*, 2008). The modulation of Group I mGluR's has shown considerable potential in both the reduction of pre-existing dyskinesia and also in attenuating the development of dyskinesia in animal models (Mela *et al.*, 2007; Johnston *et al.*, 2010; Rylander *et al.*, 2010). Drugs acting on such receptors may allow

glutamate to modulate synaptic transmission via second messenger pathways and may contribute to both NMDA-receptor dependent and independent forms of synaptic plasticity (Collingridge *et al.*, 2004; Niswender & Conn, 2010). In the striatum, the activation of group I mGluRs, particularly mGluR5, can cause potentiation of NMDA receptor-mediated responses recorded electrophysiologically from medium spiny neurons (Pisani *et al.*, 2001; Domenici *et al.*, 2003). Antagonists of mGluR5 might therefore provide an opportunity to reduce the hyperactivity of glutamatergic transmission and attenuate dyskinesia, indeed negative allosteric modulators of mGLuR5 are currently in clinical trials by Novartis and Addex (Meissner *et al.*, 2011). Other NMDA associated effects such as increased cellular Ca2+ or overactivity at AMPA receptors may also be prominent (Nash & Brotchie, 2000; Bibbiani *et al.*, 2005b). Indeed, there is evidence that dyskinesia is increased by an AMPA receptor agonist and reduced by an AMPA receptor antagonist in MPTP-treated primates (Konitsiotis *et al.*, 2000).

It therefore seems likely that glutamatergic mechanisms contribute to the expression and/ or development of dyskinesia and as such NO may be involved indirectly. Regulation of ionotropic and metabotropic glutamatergic activity appears a promising approach towards attenuating dyskinesia.

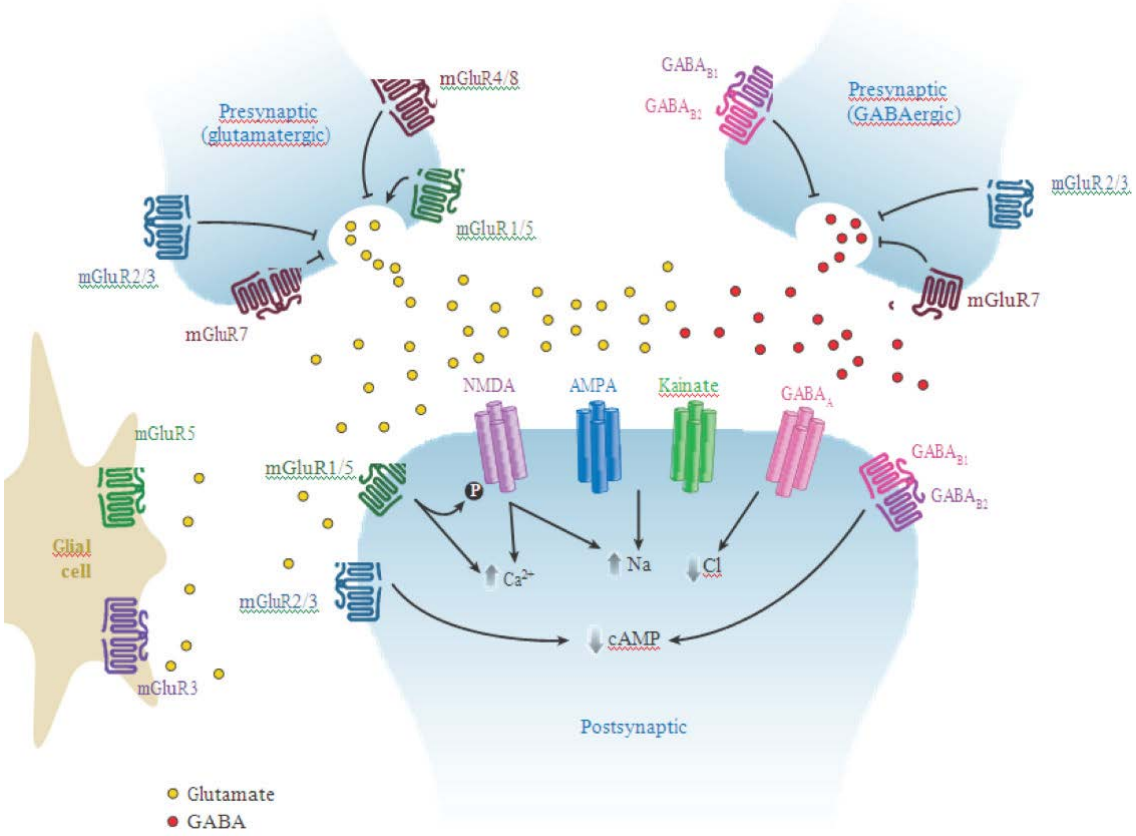

Figure 6-1 Schematic representation of mGluRs at the synapse (Niswender & Conn, 2010). Generally group I mGluRs (green) are localized postsynaptically, and group II (blue) and III (red) receptors are localized in presynaptic locations, although exceptions do occur. Group II and III receptors inhibit release of glutamate (left, yellow circles) or GABA (right, red circles), whereas group I receptors promote release when present

6.3 What does cause the priming and ongoing expression of dyskinesia?

The changes underlying dyskinesia are still not fully understood and as more evidence accumulates the picture becomes ever more complicated. One major issue in trying to understand what is happening is a discrepancy between methods employed across different laboratories, which can further distort findings. Whilst changes in 'synaptic plasticity' provide a valuable framework, and more specifically changes in glutamate pathways appear relevant in priming and expression as mentioned in section 6.2, evidence also supports other important post-synaptic alterations throughout the basal ganglia.

Altered intracellular signalling cascades appear a key factor in dyskinesia induction whereby phosphorylated forms of various proteins associated with D1 receptor sensitisation mainly concerning the direct pathway are persistently increased in the striatum following chronic L-dopa treatment (Barroso-Chinea & Bezard, 2010; Cenci & Konradi, 2010). In particular increased phosphorylation of the DA- and cAMP-regulated phosphoprotein of 32 KDa (DARPP-32) is evident at the threonine-34 residue in the 'dyskinetic' rodent striatum (Picconi *et al.*, 2003; Santini *et al.*, 2007). Furthermore increased striatal extracellular signal regulated kinases 1 and 2 (ERK1/2) phosphorylation, which can be modulated by DARPP-32, have also been demonstrated in AIMs displaying rodents treated with L-dopa (Pavon *et al.*, 2006; Santini *et al.*, 2007; Westin *et al.*, 2007). More recently Santini *et al.* (2010) have shown in a primate model that coordinated activation of cAMP/PKA/DARPP-32 and ERK is implicated in the initial priming processes underlying the emergence of dyskinesia after acute L-dopa treatment, whereas long-term administration of L-dopa leads to declining ERK activation, and persistent or even increased cAMP/DARPP-32 signalling. This decline in ERK signalling has not been seen in rodents but the discrepancy may be due to species difference or a longer period of L-dopa administration employed in the primate study.

Downstream targets of these altered signalling cascades are also implicated in dyskinesia. Increased expression of immediate early genes including *fosB*, prodynorphin, *zif268 and Arc* in nigrostriatal neurones have been demonstrated in dyskinetic rodents (Andersson *et al.*, 1999; Carta *et al.*, 2005; Sgambato-Faure *et al.*, 2005). Indeed viral vector-induced overexpression of ΔFosB enhances the ability of L-dopa to induce dyskinetic behaviour (Cao *et al.*, 2010), and *fosB* activation is closely associated with expression of the other aforementioned genes (Feyder *et al.*, 2011). In addition to transcription factors, modulation of mRNA translation by the mammalian target of rapamycin complex 1 (mTORC1) is also implicated in dyskinesia (Costa-Mattioli *et al.*, 2009). The selective inhibition of mTORC1 leads to a reduction in the emergence of abnormal involuntary movements in dopamine- depleted mice (Santini *et al.*, 2009).

Meanwhile far less is known about the role of D2-associated receptor pathways and their role in dyskinesia. D3 receptors have been shown to exert a synergistic effect on D1R-mediated transmission through direct intramembrane inter- action (Fiorentini *et al.*, 2008; Marcellino *et al.*, 2008). Accordingly, the co-treatment of L-dopa with a D3 receptor antagonist has been shown to normalise levels of membrane-bound D1 receptors in dyskinetic rats (Berthet *et al.*, 2009). There is also some evidence

showing modulation of D3 receptors can attenuate development and expression of dyskinesia in animal models of PD (Kumar *et al.*, 2009; Visanji *et al.*, 2009).

Collectively these changes may be markers of modified synaptic plasticity and could represent further angles for therapeutic intervention of dyskinesia in the clinic. Targeting downstream expression of striatal mRNA could overcome the conflicting effects associated with modulation of broader acting signalling molecules involved in dyskinesia, perhaps such as NO.

6.4 Future outlook for treatment and prevention of dyskinesia

The role of glutamate in dyskinesia has been described in some detail elsewhere (see section 6.2) and there are currently mGluR antagonists in ongoing clinical trials for this purpose, whilst the search continues for effective and clinically safe NMDA and AMPA receptor antagonists. Serotonin (5-HT) is an important modulator of dopamine release and transmission, especially following dopaminergic nerve terminal depletion, where 'false release' of dopamine from 5-HT neurones can lead to non-physiological stimulation of dopamine receptors (Carta *et al.*, 2007; Munoz *et al.*, 2008). Drugs acting on serotonergic pathways offer some promise in the treatment of dyskinesia, and agonists of 5-HT1a and 5-HT1b receptors, as well as antagonists of 5-HT2A and 5-HT2C receptors, are in development (Meissner *et al.*, 2011). The 5-HT1a agonist sarizotan was effective in reducing L-dopa induced dyskinesia in rat and primate PD models and also showed benefit in Phase II clinical trials (Bara-Jimenez *et al.*, 2005; Goetz *et al.*, 2007; Gregoire *et al.*, 2009; Marin *et al.*, 2009). However the drug failed to reach primary endpoints in two large Phase III trials, although this may be due to a prominent placebo effect or dose limitations resulting from low potency of sarizotan and partial antagonism at dopamine D2 receptors acting to worsen motor symptoms. Piclozotan, a 5-HT1a agonist, also offers agonistic properties at D3 receptors (Asubio Pharmaceuticals), and in 2011 completed a placebo-controlled, phase II, short duration proof-of-concept study, improving ON time without dyskinesia.

Adenosine A2a receptors are highly abundant in the basal ganglia, concentrated in GABA-containing medium spiny neurons of the indirect pathway, and their antagonism may contribute to restoring the altered balance in the output pathways of the basal ganglia implicated in PD (Ferre *et al.*, 1997; Jenner *et al.*, 2009). Adenosine A2a antagonists also show promise and convincing results have been seen pre-clinically whereby co-administration with L-dopa or dopamine agonists improves motor function without exacerbating dyskinesia (Kanda *et al.*, 2000; Koga *et al.*, 2000). In contrast to pre-clinical findings clinical trials with the A2a antagonist istradefylline showed a reduction in OFF-time but with an increase in ON-time with dyskinesia, although most patients showed non-troublesome dyskinesia (Hauser *et al.*, 2008; LeWitt *et al.*, 2008). The selective A2a antagonists SCH-4208140 (Schering-Plough Corp) and SYN115 (Synosia/Roche Holding AG) are currently in stage II clinical trials. V2006/BIIB014 (Biogen Idec) development is on hold in favour of 'next generation' A2a antagonists by the sponsor.

There appears to be a lack of translation of non-dopaminergic approaches to managing dyskinesia from pre-clinical studies to clinical trials. According to Meissner and collegues (2011) three major limitations have hindered the development of new treatments to date; firstly the pharmacology of dyskinesias is not fully understood. Secondly there is a lack of validated clinical outcome measures that are responsive to treatment, despite the availability of multiple dyskinesia rating scales. Thirdly, the variety of dyskinesias, their temporal patterns, anatomical distributions and associated disabilities have made the development of a unitary, sensitive and robust rating scale extremely challenging. As seems to be the case for amantadine, the only drug with proven efficacy in attenuating dyskinesia, it may be that the best symptomatic treatment for involuntary movements in PD involves modulation of multiple neurotransmitter pathways.

Ultimately dyskinesia occur due to continuing neurodegeneration accompanied by dopaminergic therapy. Therefore, rather than concentrating on their symptomatic management, an alternative and more holistic solution may come from neuroprotective or neurorestorative strategies which aim to minimise or slow down the progression of the disease itself and in so doing could reduce the chances of dyskinesia onset. Viral vector-mediated gene transfer offers the ability to enhance endogenous dopamine levels in the basal ganglia via delivery of therapeutic genes such as TH, AADC, GAD and GDNF (glial-derived neurotrophic factor)/neurturin (Wakeman *et al.*, 2011). The combined delivery of several genes in one vector is also a promising approach. Neural grafting of dopamine-secreting cells sourced from foetal tissue may also provide benefit, although graft-induced dyskinesia and emergence of Lewy bodies in grafts have compromised efficacy. There are also shortcomings with foetal tissue availability and standardisation of the grafts, but these may be overcome by employing stem-cells (Politis & Lindvall, 2012). However, it remains to be seen whether dopaminergic neurons derived from stem cells can successfully re-innervate the striatum and provide functional recovery in PD patients.

6.5 Final conclusion

The findings described in this thesis suggest that NO is not critically involved in either the expression or development of dyskinesia in PD. It is therefore unlikely that reduction of nNOS activity would afford any benefit in the clinic. This is the first time that studies have been collectively carried out into the effects of nNOS inhibitors on dyskinesia employing both the 6-OHDA-lesioned rat and MPTP-treated primate model of PD and also utilising not only the widely applied nNOS inhibitor 7-NI but also the more nNOS-specific inhibitor ARR17477. The exact mechanisms underlying dyskinesia induction and expression are still not fully understood despite considerable ongoing research effort, but as more knowledge is gained further targeted treatment by adjunctive drugs or gene therapy may provide necessary interventions to reduce or even prevent dyskinesia in the future.

Appendix

Table 0-1 Results of radioligand binding assays for ARR17477 as undertaken by MDS Pharma Services, Taiwan. Data are presented as the percentage inhibition of specific binding (% inhibition), showing the lowest concentration with a significant response. The half maximal inhibitory concentration (IC_{50}) and inhibition constant (K_i) are also presented where applicable.

Primary radioligand assay	Species	Conc.	% Inhibition	IC_{50}	K_i
Vesicular monoamine transporter (VMAT)	rabbit	1 µM	74	0.364 µM	0.302 µM
Norepinephrine transporter (NET)	human	1 µM	68	0.406 µM	0.402 µM
Adrenergic α_2, non-slective	rat	1 µM	61	0.566 µM	0.519 µM
Dopamine transporter (DAT)	human	1 µM	63	0.662 µM	0.526 µM
Monoamine oxidase (MAOB)	human	10 µM	75	-	-

(a)

Group 1

Test Day	Rat a	Rat b	Rat c	Rat d	Rat e	Rat f	Rat g	Rat h
1	D	D	D	D	D	D	D	D
2	C	B	A	B	C	A	C	B
3	A	C	C	A	B	C	B	A
4	B	A	B	C	A	B	A	C

Study i
A LD 6.25 mg/kg i.p. + ARR 6 mg/kg s.c.
B LD 6.25 mg/kg i.p. + ARR 3 mg/kg s.c.
C LD 6.25 mg/kg i.p. + ARR 12 mg/kg s.c.
D LD 6.25 mg/kg i.p. + vehicle s.c.

Study iii
A Rop 0.2 mg/kg i.p. + 7-NI 50 mg/kg i.p.
B Rop 0.2 mg/kg i.p. + 7-NI 25 mg/kg i.p.
C Rop 0.2 mg/kg i.p. + 7-NI 12.5 mg/kg i.p.
D Rop 0.2 mg/kg i.p. + vehicle i.p.

(b)

Group 2

Test Day	Rat i	Rat j	Rat k	Rat l	Rat m	Rat n	Rat o	Rat p
1	D	D	D	D	D	D	D	D
2	C	B	A	B	C	A	C	B
3	A	C	C	A	B	C	B	A
4	B	A	B	C	A	B	A	C

Study ii
A LD 6.25 mg/kg i.p. + 7-NI 50 mg/kg i.p.
B LD 6.25 mg/kg i.p. + 7-NI 25 mg/kg i.p.
C LD 6.25 mg/kg i.p. + 7-NI 12.5 mg/kg i.p.
D LD 6.25 mg/kg i.p. + vehicle i.p.

Study iv
A Rop 0.2 mg/kg i.p. + ARR 6 mg/kg s.c.
B Rop 0.2 mg/kg i.p. + ARR 3 mg/kg s.c.
C Rop 0.2 mg/kg i.p. + ARR 12 mg/kg s.c.
D Rop 0.2 mg/kg i.p. + vehicle s.c.

Figure 0-1 Modified latin-square design for treatment Chapter 3, section 3.2.4.3; Acute dopaminergic drug challenges in combination with nNOS inhibitors, treatment group 1 **(a)** and treatment group 2 **(b)**.

(a)

Test Day	Marm a	Marm b	Marm c	Marm d	Marm e	Marm f
1	B	C	D	B	A	B
2	C	D	B	C	B	A
3	D	B	A	D	D	C
4	A	A	C	A	C	D

A LD 12.5 mg/kg p.o. + CD 12.5 mg/kg p.o. + vehicle
B LD 12.5 mg/kg p.o. + CD 12.5 mg/kg p.o. + ARR 3 mg/kg s.c.
C LD 12.5 mg/kg p.o. + CD 12.5 mg/kg p.o. + ARR 6 mg/kg s.c.
D LD 12.5 mg/kg p.o. + CD 12.5 mg/kg p.o. + ARR 12 mg/kg s.c.

(b)

Test Day	Marm a	Marm b	Marm c	Marm d	Marm e	Marm f
1	B	B	A	A	B	A
2	A	A	B	B	A	B
3	E	D	E	C	D	C
4	C	E	D	D	C	E
5	D	C	C	E	E	D
6	B	B	A	A	B	A
7	F	F	F	F	F	F

A Rop 0.2 mg/kg p.o. + domperidone 2 mg/kgp.o. + vehicle
B Rop 0.2 mg/kg p.o + domperidone 2 mg/kgp.o. + ARR 3 mg/kg s.c.
C Rop 0.2 mg/kg p.o + domperidone 2 mg/kgp.o. + ARR 6 mg/kg s.c.
D Rop 0.2 mg/kg p.o + domperidone 2 mg/kgp.o. + ARR 12 mg/kg s.c.
E Vehicle + ARR 12 mg/kg s.c.
F Vehicle + vehicle

Figure 0-2 Modified latin-square design for treatment Chapter 5, section 5.4.3; L-dopa - dyskinesia expression studies **(a)** and section 5.4.4 Ropinirole - dyskinesia expression studies **(b)**.

Table 0-2 Tables of statistics (2-way ANOVA) according to figure labels.

Fig 3-4a	F	Df	(sig)
time	46.52	14	***
treatment	0.19	3	ns
interact	0.77	42	ns

fig 3-5a	F	Df	(sig)
time	29.23	14	***
treatment	0.05	3	ns
interact	1.03	42	ns

fig 3-6a	F	Df	(sig)
time	18.52	12	***
treatment	0.19	3	ns
interact	0.61	36	ns

fig 3-7a	F	Df	(sig)
time	40.19	14	***
treatment	3.16	3	*
interact	2.60	42	***

fig 3-8a	F	Df	(sig)
time	56.57	14	***
treatment	0.003	3	ns
interact	0.63	42	ns

fig 3-8b	F	Df	(sig)
time	51.74	14	***
treatment	0.36	3	ns
interact	0.50	42	ns

fig 3-8c	F	Df	(sig)
time	24.45	14	***
treatment	0.74	3	ns
interact	0.47	42	ns

fig 3-8d	F	Df	(sig)
time	67.08	14	***
treatment	0.56	3	ns
interact	0.37	42	ns

fig 3-10a	F	Df	(sig)
time	71.37	14	***
treatment	2.08	3	ns
interact	2.47	42	***

fig 3-10b	F	Df	(sig)
time	39.08	14	***
treatment	0.24	3	ns
interact	2.03	42	***

fig 3-10c	F	Df	(sig)
time	15.52	14	***
treatment	0.52	3	ns
interact	2.79	42	***

fig 3-10d	F	Df	(sig)
time	86.75	14	***
treatment	0.40	3	ns
interact	4.58	42	***

fig 3-12a	F	Df	(sig)
time	59.25	12	***
treatment	1.56	3	ns
interact	1.12	36	ns

fig 3-12b	F	Df	(sig)
time	35.01	12	***
treatment	1.13	3	ns
interact	1.02	36	ns

fig 3-12c	F	Df	(sig)
time	12.43	12	***
treatment	4.14	3	**
interact	1.16	36	ns

fig 3-12d	F	Df	(sig)
time	53.30	12	***
treatment	0.95	3	ns
interact	0.66	36	ns

fig 3-14a	F	Df	(sig)
time	88.41	12	***
treatment	3.52	3	*
interact	2.07	36	***

fig 3-14b	F	Df	(sig)
time	40.99	12	***
treatment	5.71	3	**
interact	2.56	36	***

fig 3-14c	F	Df	(sig)
time	4.33	12	***
treatment	1.73	3	ns
interact	0.91	36	ns

fig 3-14d	F	Df	(sig)
time	58.85	12	***
treatment	6.25	3	**
interact	2.00	36	***

fig 4-7a	F	Df	(sig)
time	36.78	9	***
treatment	2.53	2	ns
interact	1.61	18	ns

fig 4-7b	F	Df	(sig)
time	9.91	9	***
treatment	1.03	2	ns
interact	1.40	18	ns

fig 4-7c	F	Df	(sig)
time	10.27	9	***
treatment	0.96	2	ns
interact	1.03	18	ns

fig 4-9a	F	Df	(sig)
time	17.10	9	***
treatment	1.64	2	ns
interact	0.94	18	ns

fig 4-9b	F	Df	(sig)
time	25.36	9	***
treatment	0.45	2	ns
interact	0.85	18	ns

fig 4-9c	F	Df	(sig)
time	31.46	9	***
treatment	1.62	2	ns
interact	2.32	18	**

fig 4-11a	F	Df	(sig)
time	21.33	9	***
treatment	1.16	2	ns
interact	2.11	18	**

fig 4-11b	F	Df	(sig)
time	36.78	9	***
treatment	2.53	2	ns
interact	1.61	18	ns

fig 4-11c	F	Df	(sig)
time	23.61	9	***
treatment	0.93	2	ns
interact	0.49	18	ns

fig 4-11d	F	Df	(sig)
time	51.69	9	***
treatment	1.09	2	ns
interact	1.28	18	ns

fig 4-12a	F	Df	(sig)
time	34.10	9	***
treatment	0.99	2	ns
interact	1.68	18	*

fig 4-12b	F	Df	(sig)
time	55.14	9	***
treatment	1.34	2	ns
interact	1.89	18	ns

fig 4-12c	F	Df	(sig)
time	35.91	9	***
treatment	0.97	2	ns
interact	2.12	18	**

fig 4-12d	F	Df	(sig)
time	67.13	9	***
treatment	0.26	2	ns
interact	1.01	18	ns

fig 4-13a	F	Df	(sig)
time	29.50	9	***
treatment	1.71	2	ns
interact	2.08	18	**

fig 4-13b	F	Df	(sig)
time	62.88	9	***
treatment	4.44	2	*
interact	1.85	18	*

fig 4-13c	F	Df	(sig)
time	18.72	9	***
treatment	0.03	2	ns
interact	1.37	18	ns

fig 4-13d	F	Df	(sig)
time	105.80	9	***
treatment	9.09	2	**
interact	3.05	18	***

fig 4-15a	F	Df	(sig)
time	36.13	9	***
treatment	5.74	2	**
interact	2.43	18	**

fig 4-15b	F	Df	(sig)
time	11.90	9	***
treatment	2.13	2	ns
interact	1.24	18	ns

fig 4-15c	F	Df	(sig)
time	14.00	9	***
treatment	2.85	2	ns
interact	0.74	18	ns

fig 4-15d	F	Df	(sig)
time	37.27	9	***
treatment	4.86	2	*
interact	1.61	18	ns

fig 4-16a	F	Df	(sig)
time	63.54	9	***
treatment	3.70	2	*
interact	1.73	18	*

fig 4-16b	F	Df	(sig)
time	16.52	9	***
treatment	1.11	2	ns
interact	0.61	18	ns

fig 4-16c	F	Df	(sig)
time	15.54	9	***
treatment	1.09	2	ns
interact	1.05	18	ns

fig 4-16d	F	Df	(sig)
time	57.46	9	***
treatment	1.75	2	ns
interact	0.98	18	ns

fig 4-17a	F	Df	(sig)
time	41.70	9	***
treatment	5.34	2	*
interact	2.12	18	**

fig 4-17b	F	Df	(sig)
time	13.24	9	***
treatment	2.28	2	ns
interact	0.98	18	ns

fig 4-17c	F	Df	(sig)
time	18.48	9	***
treatment	1.98	2	ns
interact	0.69	18	ns

fig 4-17d	F	Df	(sig)
time	46.30	9	***
treatment	3.47	2	*
interact	1.31	18	ns

fig 5-3a	F	Df	(sig)
time	14.23	12	***
treatment	0.46	3	ns
interact	0.59	36	ns

fig 5-4a	F	Df	(sig)
time	32.08	12	***
treatment	1.22	3	ns
interact	1.05	36	ns

fig 5-5a	F	Df	(sig)
time	20.74	12	***
treatment	0.39	3	ns
interact	0.95	36	ns

fig 5-6a	F	Df	(sig)
time	17.81	12	***
treatment	0.20	3	ns
interact	0.48	36	ns

fig 5-7a	F	Df	(sig)
time	60.29	12	***
treatment	1.38	3	ns
interact	1.60	36	*

fig 5-8a	F	Df	(sig)
time	25.05	12	***
treatment	0.27	3	ns
interact	0.74	36	ns

fig 5-9a	F	Df	(sig)
time	2.76	8	*
treatment	0.01	1	ns
interact	0.68	8	ns

fig 5-9b	F	Df	(sig)
time	3.74	8	**
treatment	0.05	1	ns
interact	0.57	8	ns

fig 5-10a	F	Df	(sig)
time	10.64	8	***
treatment	0.01	1	ns
interact	0.55	8	ns

fig 5-10b	F	Df	(sig)
time	24.68	8	***
treatment	0.01	1	ns
interact	1.04	8	ns

fig 5-10c	F	Df	(sig)
time	10.27	8	***
treatment	0.05	1	ns
interact	0.11	8	ns

fig 5-11a	F	Df	(sig)
time	9.79	8	***
treatment	0.85	1	ns
interact	0.43	8	ns

fig 5-11b	F	Df	(sig)
time	8.01	8	***
treatment	1.04	1	ns
interact	0.74	8	ns

fig 5-11c	F	Df	(sig)
time	7.67	8	***
treatment	0.01	1	ns
interact	0.53	8	ns

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